The Efficacy of Topical Agents In the Treatment of Bacterial Biofilms: An *In Vivo* Sheep Study and an *In Vitro* Study

A thesis submitted on 7th May 2010 for

The degree of Master of Surgery

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ABSTRACT

Introduction

Recent evidence has demonstrated the presence of bacterial biofilms on the mucosa of patients with Chronic Rhinosinusitis (CRS), suggesting their role in the pathogenesis of the condition. This thesis contains two separate studies. The studies investigated novel topical therapies by using previously established *in vitro* and *in vivo* biofilm growth and detection methods. In the first study, several different proposed anti-biofilm agents were evaluated in a sheep biofilm model, each with varying degrees of immediate and short-term success against *Staphylococcus aureus* biofilms. A second study was conducted to determine the *in vitro* anti-bacterial and anti-biofilm properties of Chitosan/Dextran (CD) gel, a novel chitosan-based product with remarkable mucosal healing and haemostatic properties.

Methods

Three alternative anti-biofilm treatments: Mupirocin, CAZS (Citric Acid Zwitterionic Acid) and Gallium Nitrate were evaluated in a prospective randomized controlled single-blinded trial using a previously established sheep biofilm model of CRS. The sheep mucosal samples were analyzed for presence of *S. aureus* biofilms using BacLight staining and CLSM, and the degree of biofilm involvement was determined using FISH (Fluorescence In-Situ Hybridization).

The MIC/MBC values for CD gel and its constituents were determined by macro-dilution methods described by Jorgensen et al.[1]. Established *in vitro* biofilms grown from

common CRS pathogens (ATCC strains and clinical isolates) were subjected to treatment by CD gel and its components (chitosan and dextran). A 96-well micro-titre crystal-violet staining method described by O'Toole and Kolter [2] was used to determine the anti-biofilm profile of CD gel against several bacterial strains with known biofilm-forming capacity.

Results

Following 8 days of inoculation with *S. aureus*, all treatment groups in the sheep biofilm model showed a statistically significant reduction in biofilm surface coverage compared to no treatment. Importantly, sheep frontal sinuses treated with twice-daily mupirocin flushes for 5 days showed almost negligible biofilm growth after the follow-up period of 8 days (0.84% ± 1.25% surface area coverage per visual field).

The overall data from the *in vitro* studies suggest that CD gel has marked anti-microbial activity against planktonic and biofilm-forming bacteria. It was inhibitory and bacteriocidal at sub-clinical concentrations (25mg/mL) for all bacteria tested except for *E. coli*. When tested against a nutrient-free environment as well as a positive growth control, bacteria were essentially unable to grow in its presence.

Conclusion

Recalcitrant CRS is a difficult condition to manage and its pathogenesis has been closely linked to the presence of bacterial biofilms. Using a standardized biofilm sheep model of CRS, regular treatment with mupirocin flushes over a 5 day period showed an almost complete eradication of biofilms as assessed by mucosal surface coverage, with sustained effects over the 8 day period of follow-up.

Equally as efficacious in the *in vitro* setting, CD gel demonstrated potent anti-bacterial and anti-biofilm activity against a number of pathogenic organisms suspected of being involved in acute and chronic rhinosinusitis. CD gel's favourable haemostatic and mucosal healing profile posits it as an ideal post-ESS packing material.

These two topical agents therefore hold promise as effective treatment options in the management of CRS.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Tong Ba Le and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Tong Le#, MBBS; Theo Athanasiadis#; MBBS; Andrew Butcher, PhD; Simon Robinson, FRACS; Steve Moratti, PhD; Lyall Hanton, PhD; Peter-John Wormald MD. The Effects of a Novel Chitosan Gel on Pathogenic Nasal Bacteria. *Submitted for publication.*

#co-first authors

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PREFACE

A significant portion of the work described within this thesis has been published.

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CHAPTER 1

AIMS

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The aims of this thesis were:

1. To evaluate the efficacy of potential anti-biofilm treatments in an *in vitro* and *in vivo* animal model of Chronic Rhinosinusitis (CRS).

2. To implement the use of various modes of bacterial-biofilm detection to confirm and quantify the anti-biofilm effect. The modes of biofilm detection include LIVE/DEAD cell viability staining (Baclight, Invitrogen), quantification of Colony Forming Units, Fluorescence In Situ Hybridization (FISH) staining and Confocal Scanning Laser Microscopy.

3. In an *in vivo* sheep model of CRS, to determine the potential anti-biofilm efficacy of the novel topical treatments: Citric Acid Zwitterionic Surfactant (CAZS), Gallium Nitrate, and Mupirocin.

4. To evaluate the potential anti-biofilm efficacy of a novel Chitosan/Dextran (CD) gel, and document its *in vitro* anti-microbial profile.

5. To utilize established and reliable *in vitro* and *in vivo* models to grow and detect bacterial biofilms in order to achieve the aims outlined.

CHAPTER 2

INTRODUCTION

Chronic Rhinosinusitis (CRS)

Definition and Disease Burden

CRS is a debilitating disease of the sino-nasal epithelium characterized by persistent inflammation, with bacterial colonization and infection. In 2003, the Chronic Rhinosinusitis Task Force defined CRS as having the status of: continuous signs or symptoms for more than 12 weeks and identification of signs of inflammation on anterior rhinoscopy, endoscopy or imaging[3]. According to the National Centre for Health Statistics, 29.2 million adults in the United States were diagnosed with chronic rhinosinusitis in 2002. CRS affects 14.2% of ambulatory adults.

Pathogenesis

CRS can have a chronic, recalcitrant course often requiring multiple courses of antibiotics or surgical procedures or both. Despite its high prevalence and the severe socio-economic burden it places on society, its pathogenesis is yet to be clearly defined. Current hypotheses include the role of super-antigens, abnormalities of the inflammatory cytokine cascade, abnormal cell-mediated immune responses, and protracted osteitis of the sinus walls. Recent evidence has emerged demonstrating the presence of bacterial biofilms on the mucosa of patients with CRS, which suggests that biofilms may play a role in the pathogenesis of this condition.

Bacteriology of CRS

The pathogenesis of CRS is likely to be bacterial in origin. The bacterial profile of acute rhinosinusitis (ARS) is very different to that of chronic rhinosinusitis. This is due to the difference in physiological environment of each condition. As chronicity develops, the aerobic and facultative species are replaced by anaerobes. Whereas ARS is associated with aerobic organisms (*Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis*), the organisms predominantly isolated from CRS patients are *Staphylococcus aureus* and anaerobes (*Prevotella, Fusobacterium and Peptostreptococcus* spp)[4]. In our department, *S. aureus* is the most commonly isolated organism cultured from the mucosal swabs of CRS patients.

Cultures of individuals with CRS not responding to surgery have shown higher than usual incidences of *Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus pneumoniae,* and *Haemophilus influenzae* [5, 6].

Clinical Management of CRS

The principles of management of CRS are to restore normal mucociliary clearance by enlarging the sinus ostia and to remove the offending organism and the underlying mucosal edema by surgical irrigation, debridement and post-operative medications. This would involve endoscopic sinus surgery (ESS) and courses of antibiotics or topical corticosteroids.

It has been shown that a correlation exists between the *in vitro* biofilm-producing capacity of bacterial pathogens and unfavourable clinical evolution after ESS[7], suggesting that

biofilm-producing capacity may play a role in the pathogenesis of CRS. A comprehensive assessment by the representatives of five professional otorhinolaryngologic societies in 2004 suggested that total eradication of infection in patients with CRS would require the mechanical removal of such biofilms to adequately manage the disease[8].

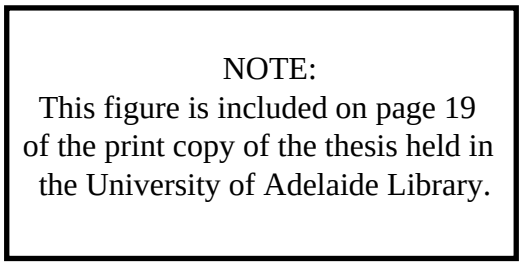
Bacterial Biofilms

Definition of Biofilms and Current Concepts

There has been an increasing body of research over the past decade on bacterial biofilms[7, 9-14]. Biofilms are a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription[15].

Figure 1 is a Scanning Electron Microscope (SEM) image of a biofilm. The arrows indicate individual cocci embedded within the exopolymeric substance (EPS) matrix. Bacteria in the biofilm phenotype are less metabolically active and more resistant to common antimicrobials. Bacteria live in biofilms as a major and possibly preferred form, and represent greater than 90% of biomass in many environments[16].

Figure 1. Scanning Electron Microscope (SEM) image of a biofilm. Arrows indicate individual bacteria embedded within the EPS matrix[17].



Ultrastructure of Biofilms

Biofilms consist of single cells and micro-colonies, all embedded in a highly hydrated, predominantly anionic exopolymeric substance (EPS) matrix. They are not a homogeneous monolayer of microbial cells on a surface, but rather, heterogeneous in both time and spatial arrangement. The cells within the deeper layers of the biofilm ultrastructure are exposed to lower oxygen tensions, and are less metabolically active. Phenotype studies have revealed that these areas within the biofilm are hypoxic and hypo-metabolic[18]. The bacterial micro-colonies are connected by well-developed channels, which convey fluid and nutrients by connective flow[19]. Polysaccharides (carbohydrate-rich polymers) and proteins constitute the majority of the EPS matrix. DNA and extracellular genetic material have also been demonstrated within the EPS[20], as well as devitalized bacterial contents[21].

The adaptive ability afforded by heterogeneous populations of bacteria within the EPS means that there is no one common biofilm structure. The adaptive response is different on inert surfaces as compared to living mucosal surfaces, which are under constant modulation by the host immune response[22].

Formation of Biofilms

Each pathway of biofilm formation is controlled by a specific cell-signaling system [21, 23-26]. The first stage involves “Attachment” and it is during this period that genetic signals are dispatched from bacteria to initiate the phenotypic changes necessary for adherence. Next, the second stage of “Adhesion and Aggregation” involves the grouping and bonding of small numbers of bacteria by structural changes in their cell walls. In high-shear environments such as on mucus-covered surfaces, the benefits of adhesion and aggregation allow successful penetration and attachment to the muco-ciliary blanket for the third stage, “EPS matrix formation”. Currently, no explicit “on-off” signals have been identified for this crucial step. It is believed to be multi-factorial in nature. The fourth stage of “Growth and Maturation” involves the redistribution of bacteria away from the substratum and the proliferation of flow channels, formation of metabolic niches and micro-colonization by phenotypic variants. Finally, as the biofilm matures the “Detachment” [27] phase is reached, characterized by embolization (by active or passive mechanisms) of the biofilm colony itself or loosely aggregated planktonic bacteria. A recent study of *P. aeruginosa* biofilms, using immunohistochemical staining and CLSM imaging, investigated the presence of an EPS matrix component (Psl) and it’s role in the dispersion of planktonic bacteria. Observing the pattern of Psl presence in each stage of biofilm development, Ma *et. al* [27] demonstrated its early influence in maintaining the architectural integrity of the matrix in the Attachment and Maturation stages, but also its absence in the centre stalk of the mushroom-like micro-colonies as they mature to create matrix cavities filled with detached bacteria, ready for dispersal and embolization. This leaves the remainder of the micro-colony structure intact.

Why do bacteria form biofilms?

There are several theories that relate to why planktonic bacteria prefer the biofilm mode of existence.

One theory suggests that bacteria form biofilms as a defense mechanism. Various factors, such as the protection conferred by the EPS matrix, the genetic heterogeneity between biofilm micro-colonies, or their slow growth rate, are conducive to their survival in physiologically inhospitable conditions. Biofilms formed in these areas can withstand physical sheer forces, nutrient deprivation, pH changes, oxygen radicals, disinfectants, and antibiotics, as well as host immune defense mechanisms. Certain bacterial strains have evolved to switch on transcription of genes required for EPS synthesis in response to certain environmental stimuli upon host entry, before the immune system mounts a specific attack[28]. For example, physiological conditions such as iron-deprivation or osmotic stress, can induce the expression of genes encoding proteins that synthesize EPS in *staphylococci* and *enterococci* as an early defensive response[29].

Another theory suggests that biofilm formation occurs as a mechanism for bacteria to remain fixed in a favourable niche. Colonization in a nutrient-rich site would promote survival of the species. This is evidenced by the production of an impressive array of bacterial surface proteins (microbial surface component recognizing adhesive matrix molecules- MSCRAMMs) that are present in the initial stages of biofilm attachment, and help bacteria adhere to the host surface upon detection of nutrient sources. These molecules are particularly abundant in *S. aureus* biofilms, and include clumping factors A and B, fibronectin binding factors and collagen binding proteins[30].

Another hypothesis suggests that biofilm bacteria exhibit cooperative behaviour to provide survival benefits as a community to maintain overall sustainability, through specialization of function[31], phenotypic differentiation, division of metabolic burden and programmed cell death[32]. This theory has recently been tested in mathematical modeling of biofilm systems [33] and appears to fit with mainstream theories of evolution.

Unique Properties of Biofilms

Many of the processes involved in biofilm formation are motivated by the requirement for survival and resistance against external destructive forces.

Biofilm-mediated infections are often chronic, are rarely resolved by host defenses, are characterized by acute exacerbations, and the microbial community is often difficult to define and culture. It is the organized functional heterogeneity that holds the key to the distinct life cycle of biofilms, and contributes to the difficulty of targeting therapies against these unique structures. The ultra-structure of biofilms also plays a significant role in protecting their survival. Surprisingly, bacteria preferentially form biofilms in high-shear environments in natural and environmental systems[15], whether the surface is rough or smooth, their EPS producing a highly visco-elastic structure. This is perhaps the reason why, in most recalcitrant biofilm-mediated diseases, complete surgical excision of the bacteria along with the surface to which it has adhered is required for definitive treatment. The EPS matrix, which encases the micro-colonies of biofilm bacteria, is composed of polysaccharides (carbohydrate-rich polymers) and proteins. Nucleotides or extracellular

DNA have also been demonstrated within the matrix, consistent with the concept of gene transfer in cell-to-cell signaling.

Established biofilms can tolerate antibiotic concentrations of up to 1000 times that needed to kill its planktonic counterparts. The precise mechanism of antibiotic resistance in biofilms remains unclear and is thought to be multi-factorial.

Earlier research into the antibiotic resistance of biofilms focused on the EPS matrix, suggesting that it retarded the diffusion of antibiotics or prevented the migration of neutrophils. It is plausible that the accumulation of beta-lactamases within the matrix could deactivate beta-lactam antibiotics [34] and infiltration of positively charged antibiotics (aminoglycosides) could be retarded by the negatively charged EPS matrix[18]. But more recent data reveal that antibiotics are able to penetrate the biofilm matrix unimpaired[35]. Other studies demonstrate the presence of inflammatory cellular elements (phagocytes and activated polymorphonuclear cells) deep within the biofilm[36].

Biofilms and Chronic Disease

In the last two decades there have been numerous studies demonstrating the presence of bacterial biofilms in chronic diseases. Chronic diseases in which biofilms have been implicated are otitis media with effusion[37], cystic fibrosis[38], cholesteatoma[39] and chronic tonsillitis[40]. Biofilms have also been isolated on prosthetic devices such as central venous catheter tips, urinary catheters, orthopedic prostheses, tracheostomy tubes[14] and tympanostomy tubes[41]. The detection of biofilms in chronic diseases, in which there is continual presentation of antigens (such as bacterial cell surface antigens and exotoxins) and persistent induction of the chronic inflammatory response, have made their implication in the pathogenesis both plausible and attractive.

It is the planktonic form of bacteria that is responsible for the patient's systemic response and manifestation of acute symptoms. The biofilm serves as a nidus of infection, periodically shedding planktonic cells to stimulate the body's defense mechanism and provoke cellular and humoral immune responses[42].

The clinical course of Chronic Rhinosinusitis (CRS) often shares this profile and bares the hallmarks of biofilm-mediated disease.

Biofilms and CRS

Recent studies have demonstrated biofilm morphology in mucosal samples from human CRS patients[10, 12, 13, 22, 43-45]. Using strict morphological criteria, Sanclement *et al* utilized Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) to demonstrate biofilm morphology on mucosal samples in 80% of their CRS patients and none of their control patients[12]. Other studies have used indirect methods of determining biofilm presence by demonstrating the *in vitro* biofilm-forming capacity of bacteria cultured from the mucosal surface of their CRS patients. Bendouah *et al*[7] represents the only study to link biofilm factors to poor clinical outcome. However, the bacterial strains investigated were cultured *in vitro* to determine their biofilm-forming capacity, and hence do not represent the phenotypes located within the biofilms. Fluorescence In Situ Hybridization (FISH) was used by Sanderson *et al*[45] to determine the presence of bacterial biofilms on the sinus mucosa of CRS patients. In this study, the mucosal samples were FISH tested for the presence of *S. pneumoniae, S. aureus, H. influenza* and *P. aeruginosa* biofilms. There was evidence of bacterial biofilms in 14 of the 18 CRS patients examined, with some specimens exhibiting poly-microbial biofilms. Interestingly, evidence of *H. influenza* biofilm was found in 2 of the 5 control patients, representing non-pathogenic colonization of the sinonasal mucosa in the asymptomatic patients. No correlation was found between the biofilms present on the sinus mucosa and the cultures taken at the time of surgery, further confirming the biofilm paradigm as a difficult bacterial entity to culture. This finding also explains why they are so recalcitrant to conventional culture-directed antibiotic therapy.

In our department, Confocal Scanning Laser Microscopy (CSLM) was used as a non-invasive, nondestructive technique to analyze fresh mucosal tissue samples for the presence of biofilms. Samples were assessed for bacterial biofilms as determined by the identification of immobile, irreversibly attached, live bacteria of appropriate size (0.5-2μm diameter) and morphology, existing in clusters and towers of micro-colonies. Of the patients who had CRS, 79% had undergone previous surgery and of these revision cases, 50% demonstrated biofilms under CLSM examination[44]. The high incidence of biofilm involvement in the refractory cases further implicates biofilms as a contributing factor to the persistence of the disease, especially when the infective nidus is not completely eradicated with surgery.

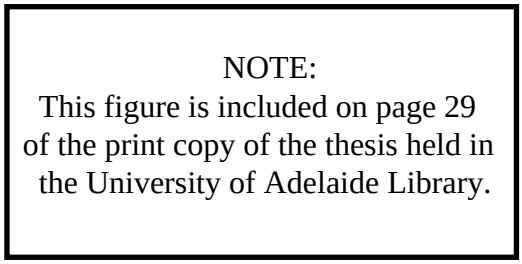
The frequency of biofilm presence in the CRS patients was 44.7%, and none in the control patients. This figure was considerably lower than those determined in previous studies of biofilms in CRS patients. The discrepancy may be explained by the difference in detection modalities used, patient population or sampling error. However, CLSM was a modality shown to offer many advantages both in this model and in a previous animal model for the analysis of biofilms in fresh tissue[17]. With CLSM, the mucosal specimens were processed fresh within 20 minutes of harvest. There were no dehydration or fixative agents used, thus conserving the architectural features of the biofilm structures. The procedure used cellular probes, making the bacterial micro-colonies more distinguishable against native mucosal structures. This, together with the freedom to analyze much larger samples compared to electron microscopic methods, makes CLSM a reliable technique for detecting bacterial biofilms.

Detection of Biofilms

The bulk of research involving biofilms in sinusitis has relied heavily on electron microscopy, in particular scanning electron microscopy (SEM) **(Figure 1).** This technique has been used to examine biofilms in human infections[9, 12, 46] and on biomedical devices[46, 47]. SEM requires the use of solvents to gradually dehydrate the specimen because the vacuum used with the electron beam is not compatible with hydrated tissue. The samples are dehydrated by a graded series of 70-100% ethanol, and then critical point dried in CO_2. The dehydration procedures result in significant distortion of architecture and artifact, particularly in mucosal tissue. Since the EPS matrix is composed of 95% water, the distortion would make SEM images not truly representative of the complex three-dimensional structure of biofilms. Another disadvantage of SEM is the fact that it only images the surfaces of tissues, thereby missing the majority of micro-colonies embedded deep within the matrix. Also, mucus and biofilm matrix are both composed of long-chained polysaccharides, and are not dissimilar in appearance on SEM analysis. This may result in incorrect identification of biofilms in mucus-filled specimens. Ha *et al* demonstrated this hypothesis when CSLM failed to confirm the presence of biofilms in 14 of 34 sheep mucosal samples previously shown to contain biofilms on SEM[17].

Transmission electron microscopy (TEM) of mucosal tissue prepared by the cryofixation technique[12] may circumvent the issue of distortion artifact, but only isolated cross sections of mucosa can be examined at any one time **(Figure 2).**
Although the EPS matrix is relatively preserved and deeper biofilms can be imaged using this technique, the restriction to single planar views limits TEM's capacity as a searching tool. This is because biofilms can be scattered at multiple mucosal sites and levels.

Figure 2. TEM (Transmission Electron Microscopy) image of a biofilm[17].



BacLight and CSLM

CSLM imaging (**Figure 3**) incorporates the use of nucleic acid probes. These probes specifically stain cellular structures and the exopolymeric substance matrix, making them distinguishable from mucus. The tissue preparartion for CSLM involves a stringent washing procedure in sterile milli-Q water to remove planktonic bacteria. Next, component A (syto 9) and component B (propidium iodide) of the BacLight LIVE/DEAD kit (Invitrogen, Molecular Probes, Eugene, OR) are added to the samples and left to incubate in darkness. When used in combination, Syto 9 preferentially stains live cells green and propidium iodide preferentially stains damaged or dead cells red (**Figures 4a,b**). Apart from their characteristic biofilm micro-colony arrangement, bacteria can be readily differentiated from other cells based on their smaller size (0.5-2μm in diameter) and more intense fluorescence (**Figure 4c**).

Figure 3. Leica SP5 Spectral scanning laser confocal microscope (CSLM), with LAS software suite (Leica Microsystems, Wetzlar, Germany).

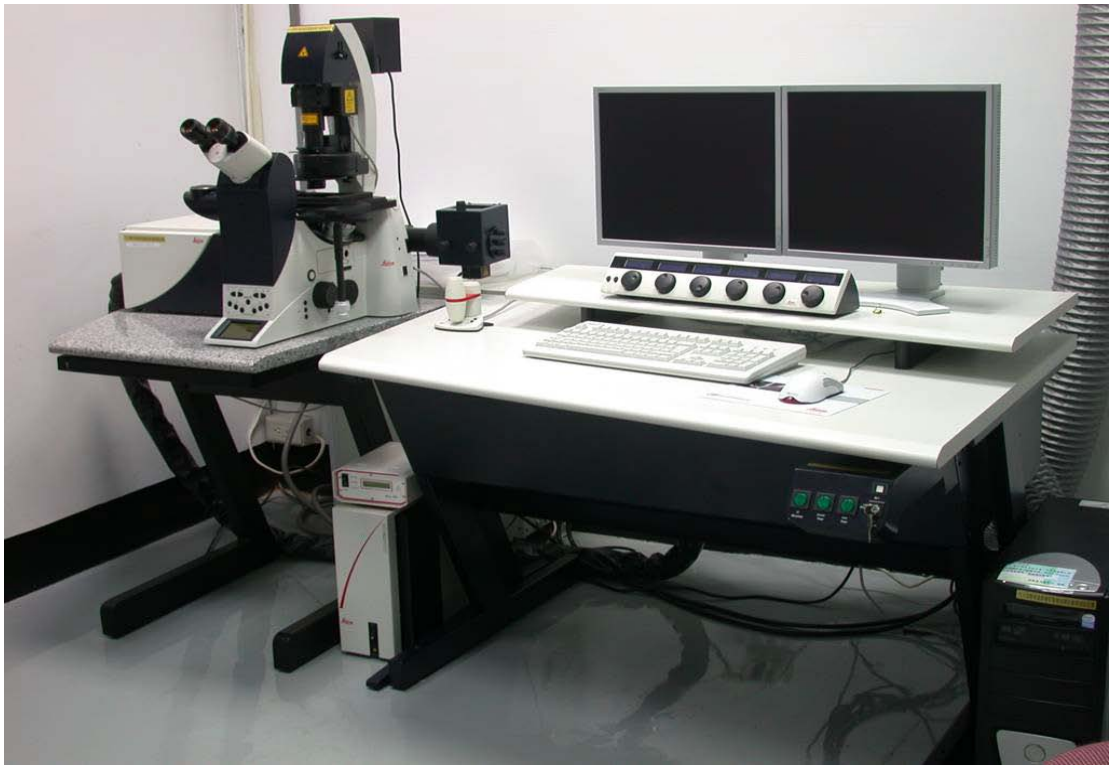


Figure 4a. CLSM image of healthy control stained with BacLight LIVE/DEAD kit (20x magnification)

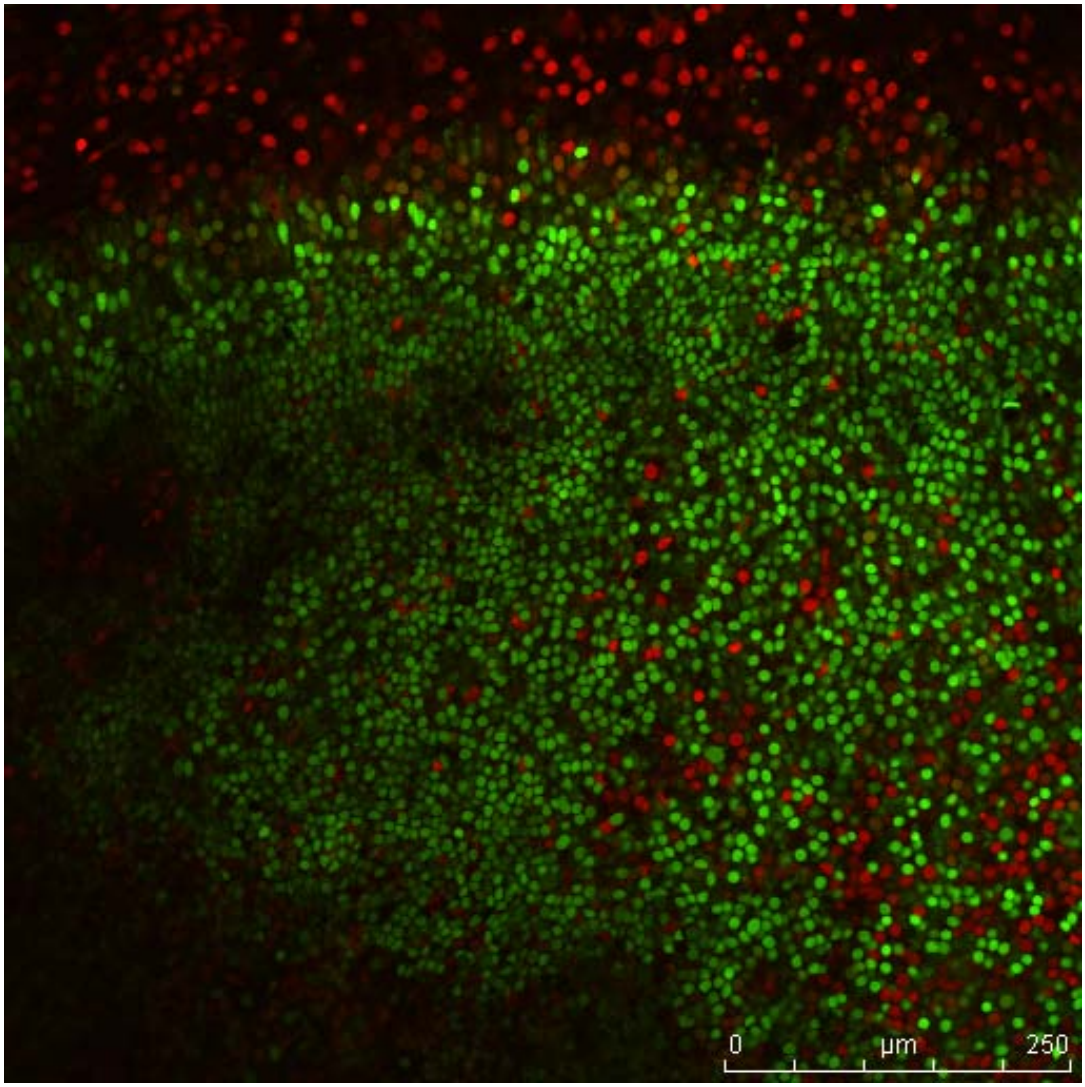


Figure 4b. CSLM image of healthy control stained with BacLight LIVE/DEAD kit (60x magnification)

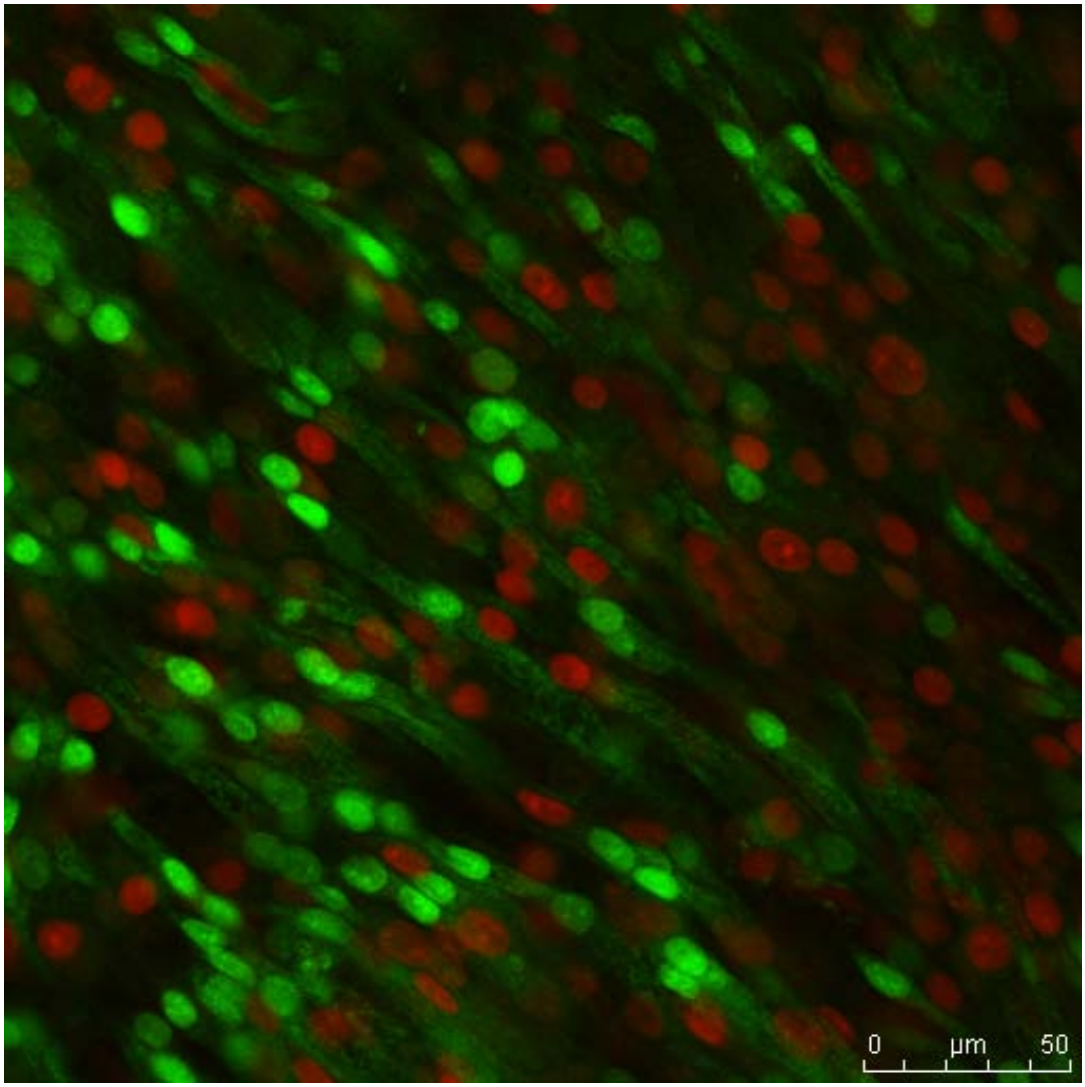
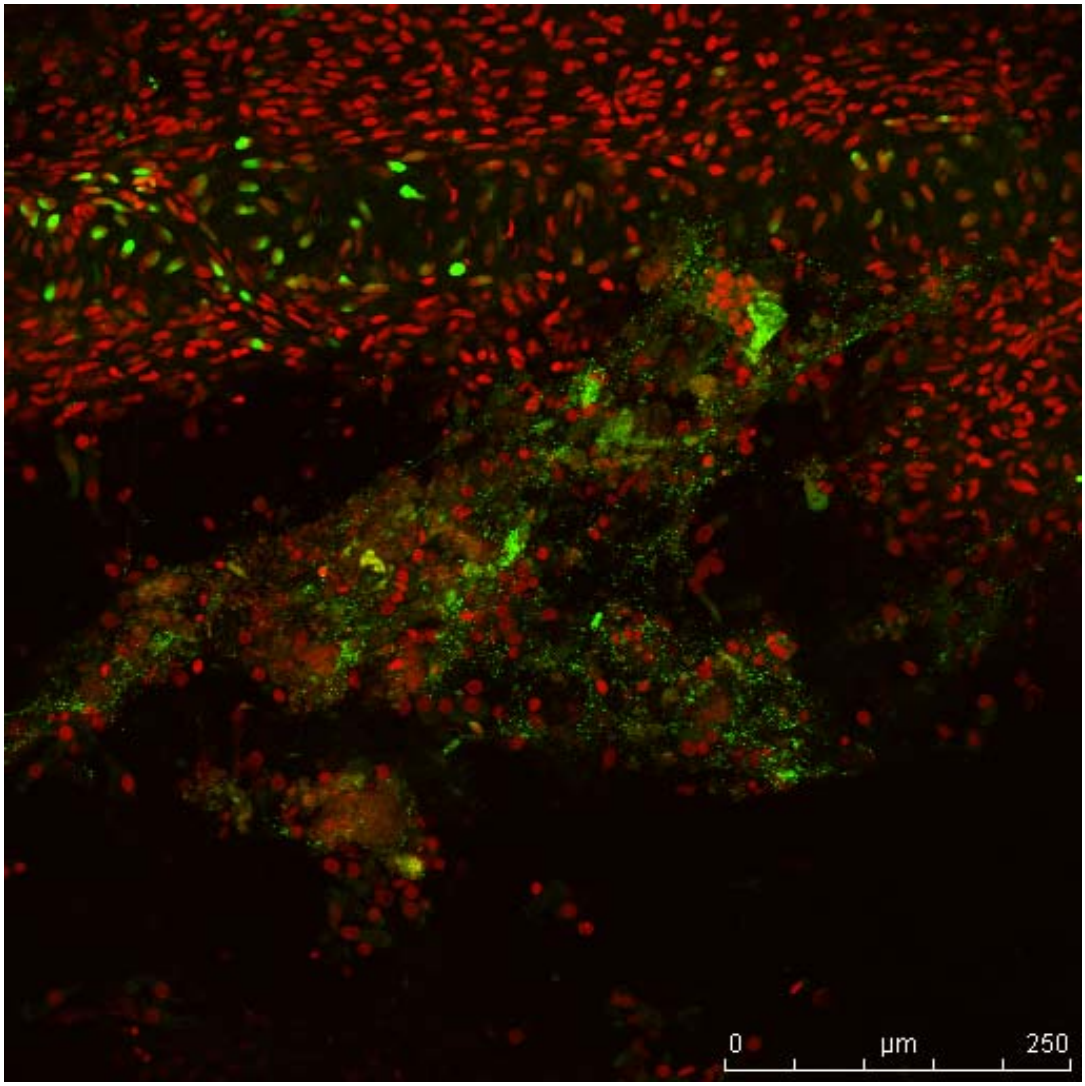


Figure 4c. CLSM image of mucosa containing *S. aureus* biofilms stained with BacLight LIVE/DEAD kit. The organized cluster of smaller, intensely fluorescent green cells represent live bacteria within a biofilm (centre of image).



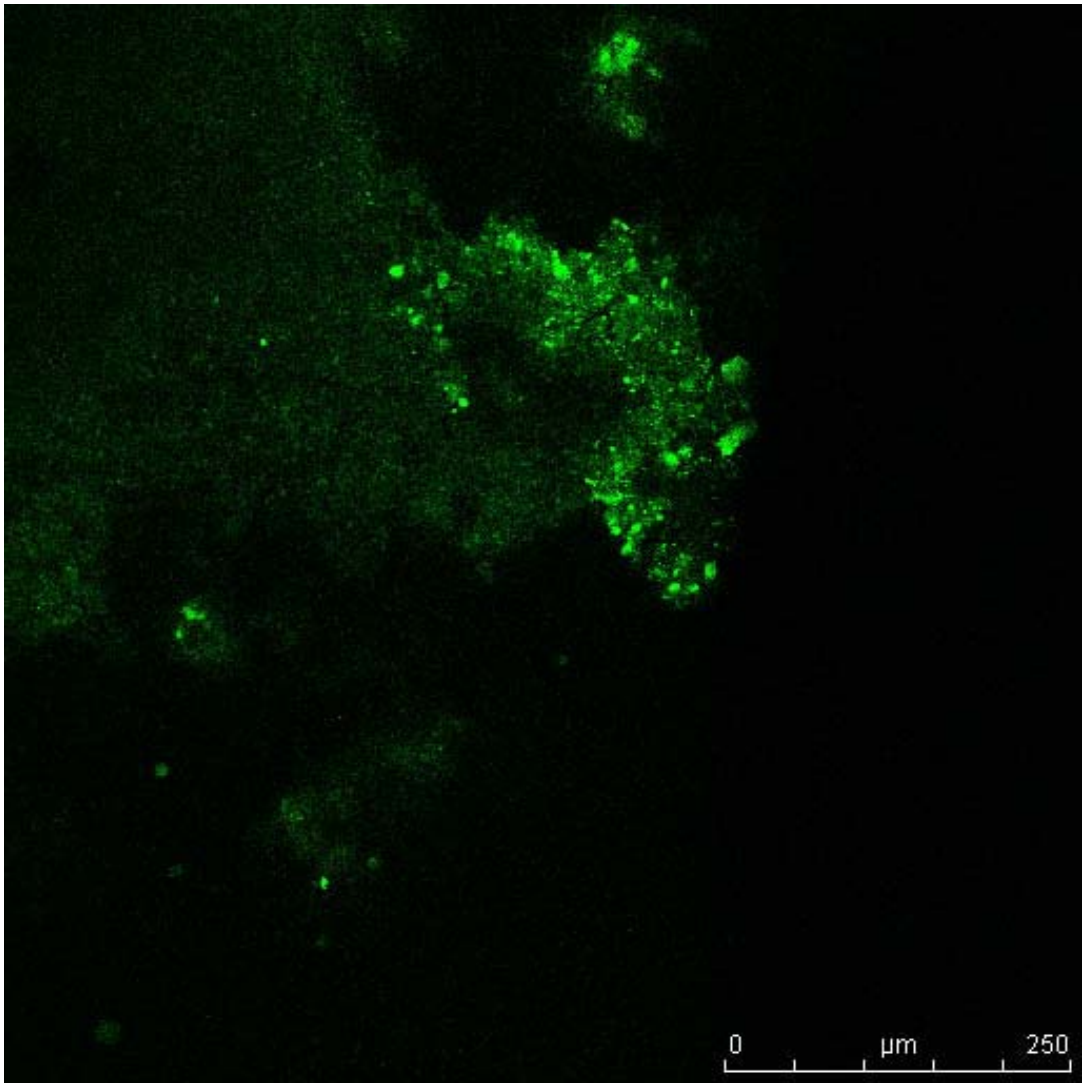
FISH and CSLM

Fluorescence In Situ Hybridization (FISH) allows the differentiation of microbial organisms within a biofilm, which sets it apart from the aforementioned detection modalities. The technique is based on the binding of fluorescently labeled molecular probes to specific RNA sequences of the target microorganism. Recently, Peptide Nucleic Acid (PNA) probes have been developed as part of a rapid identification assay for organisms such as *S. aureus*[48].

Peptide nucleic acid (PNA) molecules are pseudopeptides that obey Watson-Crick base pairing rules for hybridization to complementary nucleic acid targets (RNA and DNA). As a consequence of their uncharged, neutral backbones, PNA probes exhibit favourable hybridization characteristics for highly structured targets such as rRNA. In addition, the relatively hydrophobic character of PNA compared to other classes of DNA oligonucleotides allows better penetration of the hydrophobic cell wall of the bacteria.

The PNA FISH assay uses a PNA probe that targets a species-specific region of the 16S rRNA of *S. aureus*. Once it is bound to the *S. aureus*, visualization of the target microorganism is then made possible by CSLM, revealing multiple clusters of bright fluorescent cocci in multiple fields of view (**Figure 5**). As the EPS matrix also contains segments of RNA from cell breakdown and horizontal gene transfer, a surrounding haze of less intensity is an expected finding in the visualization of biofilms via CSLM.

Figure 5. Typical CLSM image of sheep mucosa containing *S. aureus* biofilms fluorescently labeled with Alexa-488 PNA FISH probe (AdvanDx, Woburn, MA, USA)



Using the FISH /CSLM assay, Sanderson *et al* was able to demonstrate bacterial biofilms on the sinus mucosa of CRS patients, and *H. influenzae* colonization of the mucosa of non-CRS patients[45]. Although theoretically possible, multiple probes can be used simultaneously on the same mucosal sample to demonstrate polymicrobial involvement. However, because of the laborious nature of the hybridization process and the cost of FISH probes, it would be more time-efficient and cost-efficient to focus on predominant pathogenic species.

The PNA FISH assay kit provided by AdvanDx (Woburn, MA, USA) has been validated in the rapid identification of *S. aureus* in blood cultures. Although the kit was originally designed to hybridize with *S. aureus* organisms in thinner blood smears, the same protocols were used to detect *S. aureus* biofilms in mucosal samples in the study described in this thesis. Mucosal tissue penetration of the laser used in CSLM may not have detected biofilms embedded in thicker sections of tissue, but this was an accepted limitation of the study. Thus, randomization of tissue sampling was used to minimize the impact of this factor.

Animal Model of CRS

Advantages of the sheep model

The sheep is an ideal animal model for the investigation of bacterial biofilms. Previously our department has successfully utilized the sheep CRS model to investigate various novel haemostatic agents and wound healing agents that impact on endoscopic sinus surgery in humans. More recently, Ha and Psaltis *et al* developed an ideal sheep frontal sinus CRS model for the study of bacterial biofilms that was deemed appropriate for this study[17]. Other possible animal models were explored but were not suitable technically or practically.

Dogs possess similar sinus structure to humans but were not available for ethical reasons. Pigs are dissimilar in sinus alignment and their turbinate structure makes them impractical for endoscopic surgery. New Zealand white rabbits have been used in literature[11, 49] to investigate biofilms, but have the disadvantages of smaller sinus cavities and are not certain to be free from *Pasteurella* infection.

Previous studies[11] have used the rabbit maxillary sinus, which requires an external approach to access. The open external approach requires an external incision and raising a periosteal window. This exercise would potentially disrupt the sinus mucosa within the maxillary sinus and contaminate the inoculation process. Additionally, the use of endoscopic nasal techniques cannot be replicated in these smaller animals.

With regard to sinus disease, the sheep possess a similar spectrum of conditions to humans. This includes allergic rhinitis, sinusitis and nasal polyposis. Their sinus mucosa is similar to humans histologically. Their sinus alignment and long snouts are suitable for instrumentation with slightly modified ESS equipment (**Figure 6**). The sheep frontal sinus is superficially located, making trephination for the application of topical therapies and mucosal harvesting an easy process (**Figure 7**).

Our department has accumulated vast experience with the sheep model and we have access to the facilities to support such a model[17, 50-53]. Our observation has been that the sheep are able to tolerate long operative procedures and recover from the anesthesia quickly.

Figure 6. Modified endoscopic instruments. (a) Storz 5mm straight 26cm telescope (b) 4mm straight 18cm telescope (c,d) suction tubes with and without grip plate (e)Blakesley nasal scissors (f) through-biting forceps (g) straight forceps (h) 45˚ upturned nasal forceps.

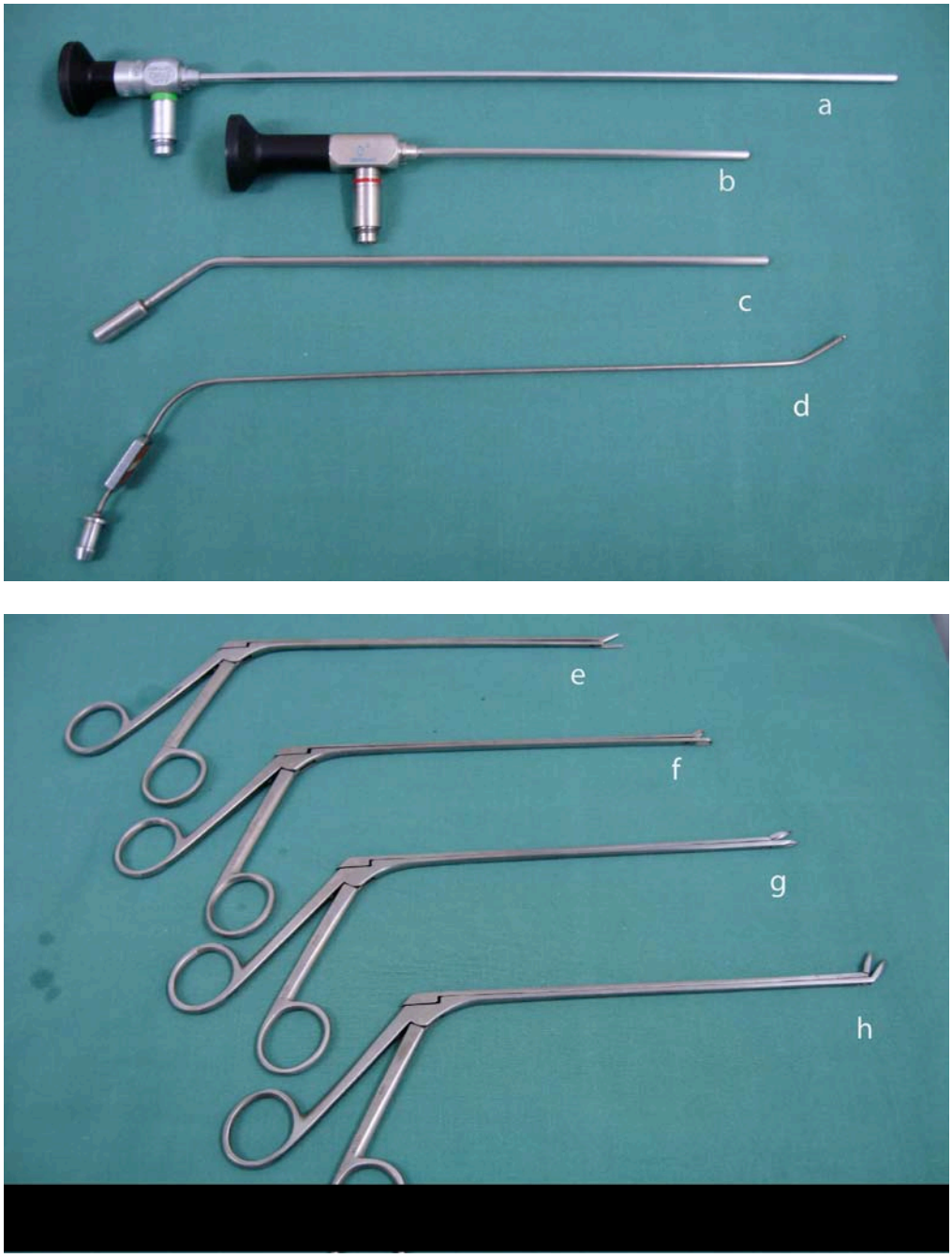
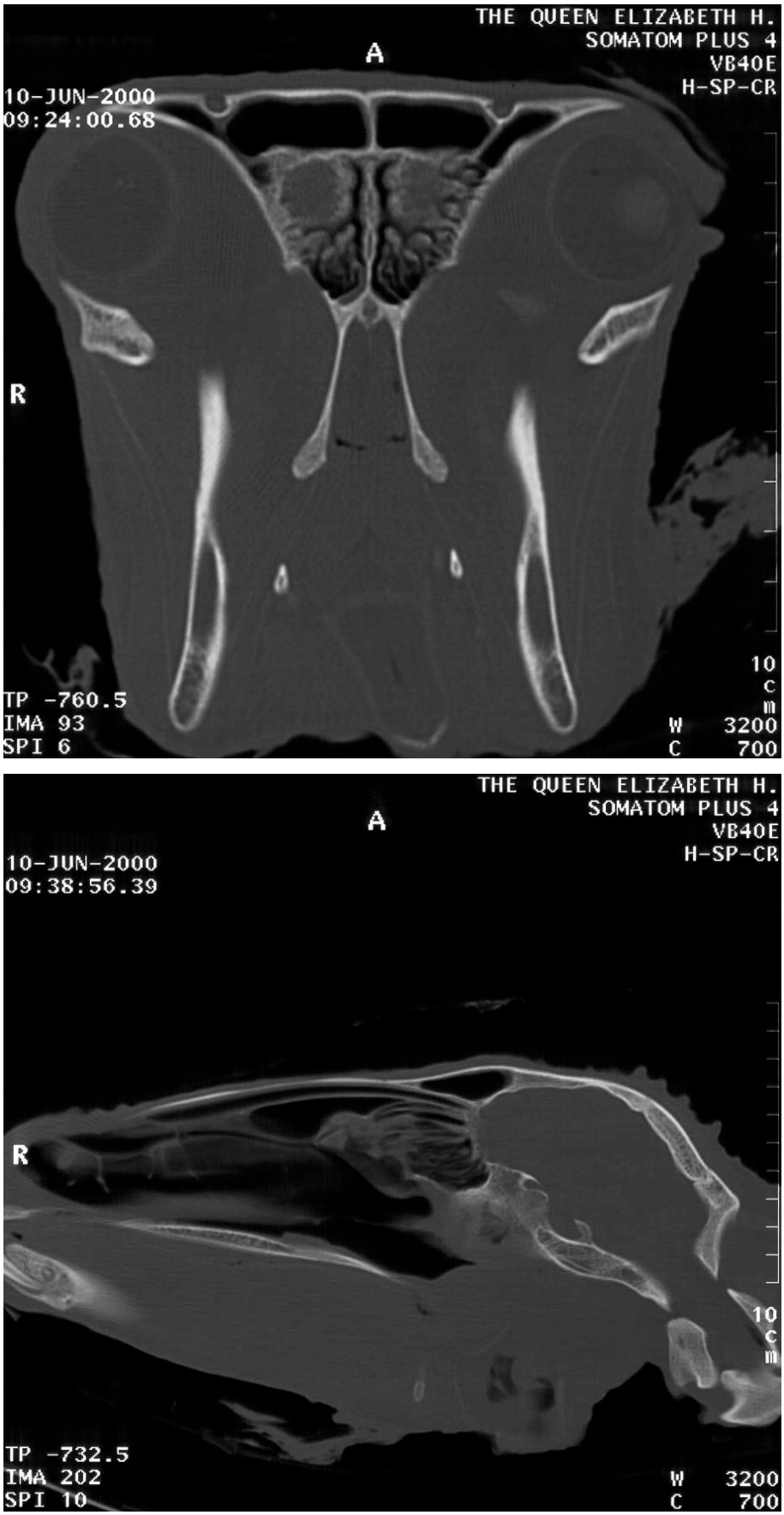


Figure 7. CT scan image of sheep sinuses in the **(a)** coronal and **(b)** sagittal planes.



Novel anti-biofilm treatments

Topical antibiotics

It is difficult to achieve effective biofilm killing concentrations in sinonasal mucosa without systemic side effects, especially when antimicrobial agents are delivered via the oral or parenteral route. The mounting evidence implicating bacterial biofilms as a mediator of recalcitrant CRS has generated multiple experimental concepts in its treatment, each with varying rates of success. Surmounting the reduced susceptibility of biofilm bacteria and robustness of biofilm-mediated infections has been the focus of many *in vitro* and *in vivo* experiments in recent literature.

Topical administration of antibiotics to the sinus mucosal membranes by either nasal irrigation or nebulizers is one novel approach that would enable higher local inhibitory concentrations to be achieved at the site of biofilm infection. This would be coupled with the advantages of low serum levels and lower risk of systemic side effects.

In vitro investigation of anti-biofilm treatments

Desrosiers *et al* explored the efficacy of topical moxifloxacin in inhibiting biofilm formation as well as eradicating established biofilms *in vitro*[54]. This group used colony forming unit (CFU) counts as a measure of bacterial viability in biofilms exposed to

increasing concentrations of moxifloxacin. Although there was no effect on mature biofilms at *sub*-MIC levels (Minimum Inhibitory Concentration), the authors reported a dose-dependent reduction in CFU counts at *supra*-MIC levels with a maximal reduction at 1000X MIC.

Chiu *et al* evaluated the effect of topical tobramycin delivered through an indwelling catheter as a regular lavage, into the maxillary sinuses of white rabbits infected with mature *P. aeruginosa* biofilms[49]. The biofilm incubation period was seven days, and the sinus lavages occurred on alternate days for seven days. At doses of 400X MIC (400μg/L), tobramycin was more effective than saline in reducing CFU counts propagated from the lavage collection fluid. However, when cultures were performed from the actual harvested mucosal samples of the sinus, there appeared to be no difference in viable bacterial counts, indicating persistence of bacterial colonization *in situ* by the attached biofilms. This is despite a reduction in the washed-out planktonic load.

Ha *et al* measured the *in vitro* efficacy of mupirocin against eight clinical isolates and one ATCC strain of *S. aureus* using the crystal violet staining model for bacterial biofilms[55]. At concentrations of 7.81 and 125μg/mL, mupirocin was able to eradicate 50% of biofilms after 1 hour of exposure. After 24 hours, 90% of the *in vitro* biofilms were eliminated. Mupirocin is an antibiotic that has broad anti-staphylococcal activity and is stable within the sinus mucosal membrane. It has very little cross-resistance and has low systemic uptake. Mupirocin, mixed in solution with Ringer's lactate, was used by Uren *et al* in a prospective clinical trial to treat surgically recalcitrant CRS patients from a tertiary rhinological treatment centre[56]. Powdered mupirocin was reconstituted into solution at a concentration of 0.05% with Ringer's lactate and used as a bi-daily nasal lavage in *S. aureus*-positive CRS patients for a period of 3 weeks. There were significantly favourable

changes seen both on endoscopic examination and noticeable symptomatic improvement after the treatment period.

Given the promising results both in the *in vitro* and clinical setting, and its safety profile, administering topical mupirocin should be considered as an alternative approach to managing surgically resistant CRS. Although effective in eradicating *in vitro* biofilms, Ha and Uren *et al* demonstrated a time-dependent killing effect of mupirocin, with results trending towards a greater rate of biofilm eradication and symptom control by regular dosing (at least 24 hours and up to a period of 3 weeks)[55, 56]. This is to ensure that any remaining nidus of bacteria cannot re-adhere and re-grow to develop into further biofilms. Such continuous antimicrobial treatment is certainly achievable with regular nasal irrigation regimes. With this concept in mind, impregnation of mupirocin into post-ESS nasal packing materials is worthy of consideration.

Loop diuretics

Using the Calgary biofilm detection assay, the loop diuretic furosemide was tested by Cross *et al* against *P. aeruginosa* biofilms and showed a 50% reduction in biofilm size at a concentration of 10mg/mL[57]. Although furosemide was not evaluated as an eradication agent, the results suggest that it could potentially be used as an adjunct to antimicrobials to destabilize biofilm structures and assist in their eradication.

Surfactants

Chemical surfactants are amphiteric molecules that act to modulate the way other molecules behave at interfaces and in solution. The amphiteric properties of surfactant allow it to be solvent in both water and organic substrates. By altering the mucus-mucosal surface interface, chemical surfactants have the potential to act as mucoactive agents to assist with mucociliary clearance and prevent biofilm adherence. Additionally, surfactants can disrupt cell membrane integrity, increase cell membrane permeability causing metabolite leakage, and can interfere with cell membrane exchange functions. This would ultimately disrupt bacterial cell survival mechanisms to control biofilm growth.

Chiu *et al* explored the use of surfactants as agents to disrupt biofilm integrity in a prospective, non-randomized clinical study of post-operative ESS patients[58]. After self-irrigating their nasal cavities with 1% baby shampoo diluted in normal saline for a period of 4 weeks, the subjects reported a 46.6% rate of improvement in their Sinonasal Outcome Test 22 scores and a 63% rate of improvement in their olfactory function along with endoscopic evidence of healthier mucosal function (decreased rate of mucosal edema and polypoid degeneration).

Despite the theoretical advantage of chemical surfactant, the removal of adherent biofilms would also require disruption of the EPS matrix that confers protection to the bacterial micro-colonies. Strong calcium-ion bridges that bind the long polymeric chains in the EPS matrix are partially responsible for the integrity of biofilm structures. The disruption of these “cross-links” by a sequestering agent, citric acid, followed by dissolution of the unbound chains by surfactant, was a strategy posited by Desrosiers *et al*[59]. This group examined the effect of CAZS -citric acid and zwitterionic surfactant (caprylyl sulfobetaine) -on 3 day-old *in vitro S. aureus* and *P. aeruginosa* biofilms grown in drip flow reactor

(DFR) systems to mimic the low shear environment in which robust biofilms flourish. Along with static CAZS treatment, hydropressure, at 31.4 psi, was also examined as a delivery method for the CAZS in the *in vitro* experiment. Based on the CFU counts obtained from viable bacteria cultured from the DFR slides, both static CAZS and hydrodynamically treated biofilms showed a reduction in bacterial load. However, hydrodynamic treatment with saline also produced a reduction in biofilms, but to a lesser extent (2.3 log reduction in *S. aureus* biofilms for saline, as compared to 2.5 log reduction for static CAZS and 3.9 log reduction for hydrodynamic CAZS).

Hydropressure

Hydropressure has been used with success in orthopedic surgery as a method of mechanically removing biofilms from metallic surfaces prior to prosthetic implantation[60, 61]. The effect of jet lavage applied to a surface lined with mature biofilms can reduce the CFU count by 100 fold, with an increased effect when a surfactant is added to the irrigation fluid[62].

Gallium Nitrate

Iron (Fe) metabolism is integral to the pathogenesis of bacterial infection, particularly chronic infection[63]. In almost all pathogens, Fe is essential for the development and functioning of key enzymes such as those involved in DNA synthesis and electron transport. A high Fe concentration *in situ* promotes the growth of bacterial biofilms, because high Fe levels are required for the formation of cell clusters early in biofilm

development and maturation[64]. Many host defense mechanisms result in the sequestration of Fe to limit the spread of bacterial growth by lowering the Fe concentration locally. Data from a number of laboratory studies have shown the importance of Fe limitation in blocking acute infection from various bacterial species[63, 65]. Fe metabolism is a major vulnerability of pathogenic bacteria. Gallium (Ga^{3+}) nitrate has been used to exploit the vulnerability because this transition metal is nearly identical in ionic radius to Fe^{3+}, effectively acting as a "Trojan Horse" to disrupt Fe-dependent processes. Unlike Fe^{3+}, Ga^{3+} cannot be reduced, and it is the sequential oxidation and reduction reactions that are critical for many of Fe's biological functions[66].

Importance of Mucosal Injury, Restoration of Normal Mucociliary Clearance in treating CRS

With any therapy, the mucosal epithelial functioning must be preserved because adequate mucociliary clearance remains the best treatment to prevent CRS.

If it is clear to the clinician that conventional systemic antimicrobial and anti-inflammatory agents have not adequately controlled the disease, then surgical ventilation of the involved paranasal sinuses is the mainstay of treatment. This involves a combination of restoring mucociliary clearance and surgically removing bacterial biofilms from the sinonasal mucosa to prevent future exacerbations caused by re-infection by a viable remnant nidus of bacteria.

Post-operative mucosal healing is integral to the control of CRS symptoms. Patients with rhinosinusitis recalcitrant to standard medical and surgical therapy often show rapid disease recurrence after an initial response to antibiotics. Surgically recalcitrant CRS patients represent some of the most challenging patient cohorts in any tertiary rhinology

practice. Numerous studies have tested the adequacy of post-ESS packing materials to inhibit bleeding, the development of adhesions, and positively impact on the healing of the ciliated mucosal tissue. Adhesions remain the main cause of failure in ESS, and poor mucosal healing is a significant contributor to this failure. This is particularly relevant to more radical surgery such as the modified endoscopic Lothrop procedure. Maintaining patency of the neo-frontal ostium and eradication of infection, are paramount to ensuring the success of the procedure. In examining mucosal samples of recalcitrant CRS patients requiring multiple ESS procedures, Psaltis *et al* identified the presence of biofilms in this particular group by CSLM criteria[44].

Very few studies have explored the effect post-operative packing materials may have on the re-generation of bacterial biofilms. Packing materials with anti-biofilm properties may offer the ideal medium for exerting sustained antimicrobial effect on remnant biofilms or prevent further re-growth of bacteria by providing a hostile anti-biofilm environment within the sinonasal cavity.

Post FESS packing material

Chitosan and Mucosal Healing Studies

Chitosan is a natural polymer obtained from chitin, which is the structural element in the exoskeleton of crustaceans and squid. It has been studied extensively for its potent haemostatic properties, but has recently been investigated as an anti-adhesion agent in general surgery[67, 68]. When combined with dextran derivatives, chitosan forms cross links to form a mucoadhesive gel (Chitosan-Dextran or CD gel). Bleeding and wound healing are intimately related. The initial inflammatory phase of wound healing involves haemostasis and clot formation. The injured tissue induces fibroblast migration, resulting in collagen deposition and fibrous adhesion formation. Athanasiadis *et al* studied the effect of CD gel on full thickness mucosal injuries created in the lateral nasal wall of sheep, specifically observing the effect on mucosal re-epithelialisation, re-ciliation and ciliary beat frequency[52]. The most significant effect on wound healing was CD gel's ability to enhance the recovery of epithelium, reflected in the significantly greater degree of re-epithelialisation and percentage surface area that was re-ciliated 28 days post-injury. These parameters were compared to other commercially available haemostatic agents recombinant tissue factor (rTF), poly-ethylene glycol ("SprayGel") and saline control. Overall, CD gel demonstrated a superior wound healing profile with 70% re-epithelialisation and 62% reciliation of surface area 28 days post-application.

CD gel is a novel and unique synthetic gel that can be used as a post-ESS mucoadhesive dressing, but it also has the ability to dissolve over a period of 7 to 9 days. Its ability to

adhere to the sinonasal mucosa makes it an ideal carrier for a number of antibiofilm agents. Previous studies have shown that chitosan itself may have anti-biofilm properties[69-72].

CHAPTER 2

The efficacy of topical antibiofilm agents in a sheep model of rhinosinusitis

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The efficacy of topical anti-biofilm agents in a sheep model of rhinosinusitis

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Short title:
Effects of anti-biofilm treatments in a sheep model of rhinosinusitis

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Key Words:
Mupirocin, citric acid, zwitterionic surfactant, biofilms, sheep, gallium nitrate, chronic rhinosinusitis, confocal, scanning, microscopy, fluorescence in situ hybridization, anti-biofilm, treatment.

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The efficacy of topical antibiofilm agents in a sheep model of rhinosinusitis.

American Journal of Rhinology 2008 Nov-Dec;22(6):560-7.

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I hereby certify that the statement of contribution is accurate.

Signed

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Assisted analysis of samples, data interpretation and manuscript evaluation.

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Tan, L.W.

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The efficacy of topical antibiofilm agents in a sheep model of rhinosinusitis.

American Journal of Rhinology 2008 Nov-Dec;22(6):560-7.

Wormald, P.J.

Supervised development of the study, helped in data interpretation and manuscript evaluation and acted as corresponding author.

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…….Date……

Le, T., Psaltis, A., Tan,L.W. and Wormald, P-J. (2008) The efficacy of topical anti-biofilm agents in a sheep model of rhinosinusitis.
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CHAPTER 3

The effects of a novel chitosan gel on pathogenic nasal bacteria

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The effects of a novel chitosan gel on pathogenic nasal bacteria

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Short title:

Effects of a chitosan gel on nasal microbes

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Key Words:

Endoscopic sinus surgery, chitosan, dextran, wound healing gel, chronic rhinosinusitis, biofilm, anti-microbial, minimal inhibitory concentration, minimal bactericidal concentration, sealant, adhesion barrier.

STATEMENT OF AUTHORSHIP

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Athanasiadis, T.

Assisted analysis of samples, data interpretation and wrote manuscript.

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…….Date……

Butcher, A.R.

Data interpretation and manuscript evaluation.

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STATEMENT OF AUTHORSHIP

The effects of a novel chitosan gel on pathogenic nasal bacteria

Wormald, P.J.

Supervised development of the study, helped in data interpretation and manuscript evaluation and acted as corresponding author.

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Signed …….Date……

ABSTRACT

Introduction

Chronic Rhinosinusitis (CRS) has a complex pathogenesis. Bacterial biofilms have been associated with poorer outcomes post endoscopic sinus surgery (ESS). A novel absorbable chitosan dextran gel has been shown to reduce the rate of post-ESS adhesion formation, bleeding and may have anti-biofilm properties.

Method

The antimicrobial profile for chitosan/dextran (CD) gel and its constituents were determined using a macrodilution method of evaluating MIC/MBC, zones of inhibition of growth, colony-forming units for evaluation of microbial growth activity. The susceptibility tests were performed for common CRS pathogens as well as clinical isolates. The antibiofilm effects of the test agents were assessed using a 96-well micro-titre crystal violet staining method described by O'Toole and Kolter. Only organisms of biofilm-forming capacity were included in the analysis.

Results

All bacterial strains tested were susceptible to dextran. CD gel was both bactericidal and inhibitory to all strains except for *E. coli*. Overall, dextran exhibited the greatest effect on growth inhibition with a slightly lesser inhibitory effect demonstrated by CD gel on zone of inhibition analysis and microbial growth activity tests. More importantly, neither CD gel nor its constituents promoted growth of tested strains compared to no-treatment. At subclinical concentrations (25mg/mL) CD gel produced significant reduction in biofilm formation for all tested bacteria apart from *S. pneumoniae*.

Conclusion

Overall, CD gel demonstrated marked antimicrobial activity against a wide range of planktonic and biofilm forming bacteria. The data support randomized clinical trials that demonstrate CD gel's remarkable ability to reduce the appearance of infection in the nasal cavity compared to control. This, combined with its unique mucosal healing properties, posits CD gel as a useful absorbable nasal packing material post-ESS.

INTRODUCTION

Chronic rhinosinusitis (CRS) has a complex pathophysiology with a number of possible aetiologies postulated.

These include bacterial biofilms[43], staphylococcal superantigens[96], fungus[97], abnormal cell-mediated immune responses and abnormal cytokine cascades[98], prolonged sinonasal osteitis[99] as well as anatomical predisposition[100, 101]. These aetiologies are thought to result in mucosal inflammation and increased secretions that can block sinus ostia and drainage outflow tracts. The environment is then ideal for secondary bacterial growth and resultant mucosal trauma which propagates the cycle of oedema, thickened mucosa and disruption of the sinonasal mucociliary system[102].

In acute sinusitis, organisms such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* are commonly isolated[103]. Staphyloccocus is one of the most commonly cultured organisms from CRS patients' although it is not necessarily pathogenic in all instances[104, 105]. In chronically infected sinuses the main aerobes cultured include Gram negative bacilli such as *H. influenzae*, *K. pneumoniae*, *E. coli* and *P. aeruginosa*[106]. As the disease process becomes more chronic, the aerobic and facultative bacteria are gradually replaced by anaerobes such as *Peptostreptococcus* species, *Prevotella*, *Fusobacterium*, *Enterobacter* species and *Bacteroides fragilis*[106-109].

Recently biofilms have been described on the sinus mucosa of patients with CRS[12, 43]. Importantly biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* have been

found to be associated with a poor prognosis following endoscopic sinus surgery (ESS)[7, 110].

Recently non-absorbable nasal packing following ESS has been been superseded by absorbable packing materials. However, these packs have been shown to be largely ineffective in hemostasis and adhesion prevention [111, 112] with some authors suggesting that no packing is the best alternative[113]. A recent development of a novel absorbable haemostatic chitosan gel has shown promising results in both hemostasis and wound healing following endoscopic sinus surgery[114].

Chitosan has been reported as having a wide spectrum of antibacterial[115], antiviral[116], antifungal[117, 118] and even antibiofilm[71, 119, 120] activity. Use of chitosan has also been shown to prevent post operative infection developing in highly contaminated wounds in mice[121]. Due to the risk of infection from foreign material being placed in the nose as well as possible colonization by pathogenic CRS microbes, the effects of the gel on known and potential nasal pathogens warrants investigation.

The aim of this study was to determine the antibacterial and antibiofilm properties of this novel Chitosan-Dextran (CD) gel with reference to some well known ATCC strains as well as clinical isolates cultured from CRS patients.

METHODOLOGY

The minimal inhibitor concentration (MIC) of constituent components of the Chitosan-Dextran (CD) gel (dextran and chitosan) against bacterial organisms was determined using a broth macro-dilution method described by Jorgensen *et al* [1], modified to suit the appropriate bacterial strains commonly found in CRS.

Antimicrobial components

Chitosan (alone) was prepared fresh by dissolving 0.5g of the sterilized dry product in 10mL of sterile water 20 minutes before testing. The stock solution of chitosan was added to 10mL of broth to give a final stock antimicrobial concentration of 50mg/mL. The antimicrobial agents were tested at $\log_2$ serial dilutions. From the final stock tube, 1 mL was removed and transferred into the next tube containing 1mL of broth. After mixing, 1mL of this dilution was removed and transferred in the next tube until a final concentration of 0.10mg/mL was achieved. The same procedure was repeated for dextran. To form CD gel the separate components (chitosan and dextran) were mixed together.

Inoculum

Isolates were inoculated into a broth that would support good growth. Mueller-Hinton broth (Oxoid Australia, Thebarton, South Australia) was used for *Staphylococcus aureus* *Pseudomonas aeruginosa* and *Escherichia coli*. CSF broth (Oxoid Australia, Thebarton, South Australia) was used for *Haemophilus influenzae* and *Streptocuccus pneumoniae*. ATCC strains of *S. aureus* (25923), *S.pneumonia*e (49619), *H. influenza*e (49247) and *E.*

coli (35218) were used for susceptibility testing. One clinical isolate of *P. aeruginosa* was chosen. A few colonies of each isolate grown overnight on agar plates were directly suspended in broth until the adjusted turbidity matched a 0.5 McFarland standard. A portion of the standardized suspension was diluted 1:100 (10^6 CFU/mL) with broth. One mL of this final inoculum was added to each tube containing 1mL of the diluted compound in broth to achieve a final concentration of 5 x 10^5 CFU/mL. Broth without test compound was inoculated with bacteria and used as positive growth control. Each tube was incubated in air at 35^0C +/- 2 ^{0}C for 16-20 hours and evaluated for growth turbidity the following day.

Analysis

The MIC was determined as the anti-microbial concentration that visibly inhibited growth of the organism, by evaluating the turbidity of each tube's contents with the unaided eye. Assessment of Minimal Bacteriostatic Concentration (MBC) was carried out by sub-culturing 100 µL from each no-growth tube onto Horse Blood Agar plates (Oxoid Australia, Thebarton, South Austrlia) and incubating overnight in air at 35^0C +/- 2 ^{0}C. MBC was determined as the lowest dilution showing no growth.

Susceptibility Method by Disk Diffusion

Antibacterial activity using agar diffusion method was performed by inoculating a Mueller Hinton Agar plate or Mueller Hinton Agar plus Sheep Blood (Oxoid Australia, Thebarton, South Australia) with a 0.5 McFarlands standard of the test organism according to standard antimicrobial susceptibility testing procedures[1]. Seven 5 mm holes were then cut into the

agar using a sterile boring tube and the agar plugs removed. The wells were filled with 100 uL of the test compounds in duplicate (chitosan, dextran, chitosan gel) at concentrations of 5% as well as a control well filled with 100 uL of sterile 0.9% saline. All plates were then incubated for 24 hours at 35^0C aerobically, anaerobically or microaerophilically depending on the optimal growth conditions of the test organism. At the end of the incubation any zone of inhibition was measured and recorded.

Microbial growth activity

The ability of the test compounds (chitosan, dextran and CD gel) to support bacterial growth, hold a bacteriostatic state or show antibactericidal activity was assessed by inoculating 1 ml of 5% concentrations of the test compounds with 1 uL of a 0.5 McFarlands standard suspension of the test organism (approximately 1.5×10^5 organisms per mL). These tests were performed in triplicate.

Non-nutrient controls (sterile 0.9% saline) and growth controls (CFS broth, Oxoid Australia, Thebarton, South Australia) were also inoculated with the same bacterial suspension and all tubes were incubated for 24 hours at 35ºC aerobically or microaerophilically depending on the optimal growth conditions for the test organism. After incubation, 1 uL test compound, saline or CSF broth suspension was removed from each tube and lawn plated onto Blood or Chocolate agar (Oxoid Australia, Thebarton, South Australia) and then incubated for 24 hours at 35ºC under aerobic or microaerophilic conditions depending on the test organism. Following incubation, digital photographs of the agar plates containing colony forming units (CFU) were taken and later manually counted with digital marking using image editing software (Photoshop, Adobe). These were then compared with the non-nutrient sterile saline control tube.

In-vitro anti-biofilm properties.

To determine the action of CD gel and its constituents against in-vitro biofilms of various bacterial reference strains and clinical isolates appropriate for CRS, a 96-well micro-titre crystal-violet staining method was used. Our modified procedure was based on the method described by O'Toole and Kolter[122]. The organisms were prepared as described above for the macro-dilution method, the final desired inoculum concentration being 5×10^5 CFU/mL. The organisms tested included the same ATCC strains used in the macro-dilution method, as well as 4 clinical isolates of *S. aureus* from patients with CRS according to the criteria of the CRS Task Force 2003[123]. All organisms used were initially examined for biofilm forming capacity with the crystal violet method as well as confirmation via four chamber slide analysis using confocal scanning laser microscopy (CSLM) as previously described[55]. Only organisms that formed visible biofilm via CSLM monitoring were included in the analysis.

Prevention of biofilm growth

One row of wells was filled with 100µL of CD gel (50µL of chitosan mixed with 50µL of dextran) and allowed to dry for 15 minutes. The solidified gel was then evenly spread with a sterile micro-pipette tip to ensure complete coverage of the walls and base of the well. 100µL of broth was added, along with 10µL of inoculum. The bacterial strains were evaluated in replicates of three at day 8 and the experiment repeated twice. Negative controls included CD gel coated wells with broth, without any addition of bacteria. Positive controls included broth and bacteria without any test agent.

Inhibition of established biofilms

Two rows of wells were filled with 200µL of broth and 10µL of inoculum and allowed to establish biofilm growth for a period of 8 days in 35ºC. On alternate days, all wells underwent media change. 50µL of media was aspirated from each well using sterile micro-pipette tips and replaced with 50-150µL of fresh media to prevent dehydration as this has previously been shown to allow maximal biofilm growth[55]. At day 8, once biofilms had established, the wells were treated separately with 100µL of dextran and 100µL of chitosan for 24 hours. A final row of wells contained untreated inoculated broth and was used as positive growth controls.

Analysis of 96 well plates was conducted using a crystal violet assay and BioRad microplate reader (Hercules, CA) as previously described[55]**.**

Permanox chamber slide CLSM analysis

The biofilm forming capacity of each reference strain was confirmed using Permanox chamber slides (Nunc, Roskilde, Denmark) and confocal laser microscopy (Leica Microsystems, Gretzwald) in a technique previously described[55].

Statistics

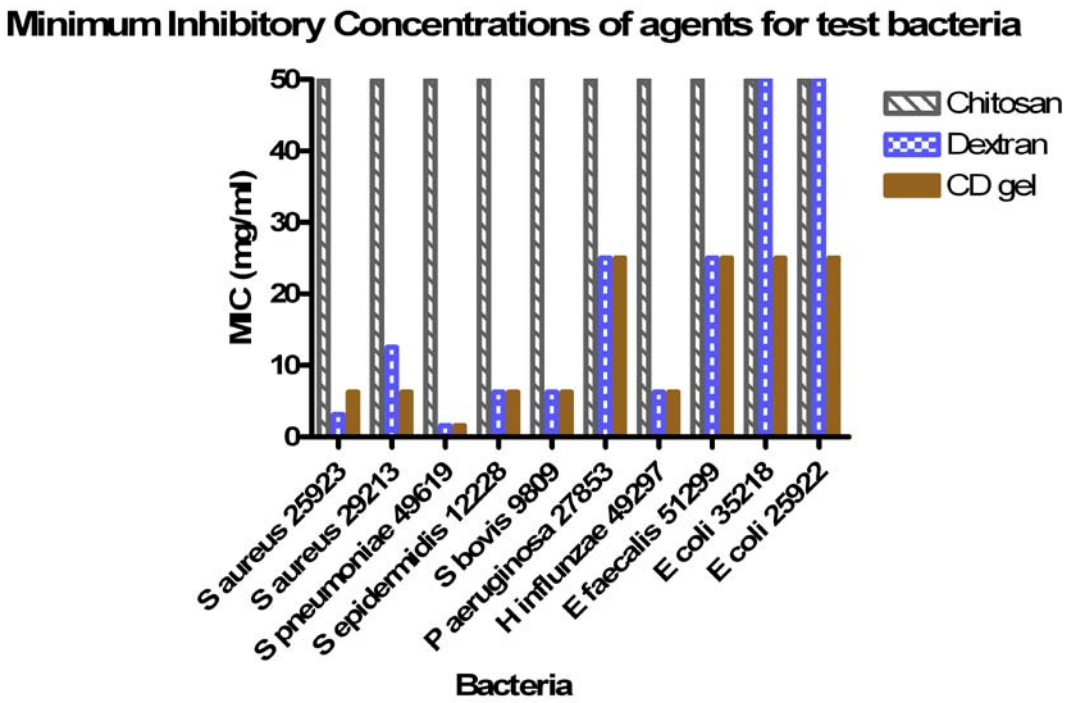
Statistical analysis was supported by an independent statistician provided by Statistics South Australia. Prevention of biofilm formation and inhibition of established biofilms were analysed using 2 way ANOVA with Bonferroni post test correction for multiple testing (GraphPad Prism).

RESULTS

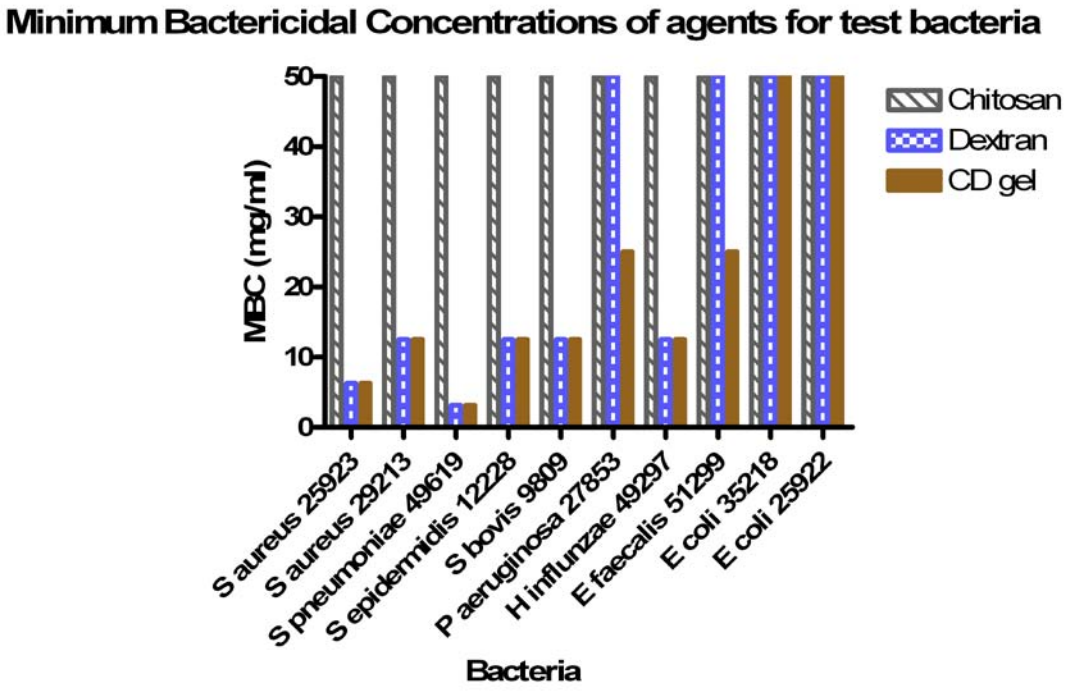
MIC/MBC

MIC and MBC of each test agent for each bacterial strain are displayed in **graph 1** and **graph 2**. Apart from *E. coli* and *P. aeruginosa*, all bacterial strains tested were susceptible to dextran. In combination form, CD gel was both bactericidal and inhibitory to all strains except for *E. coli*. None of the bacteria tested were susceptible to chitosan at sub-clinical concentrations (50mg/ml).

Graph 1.



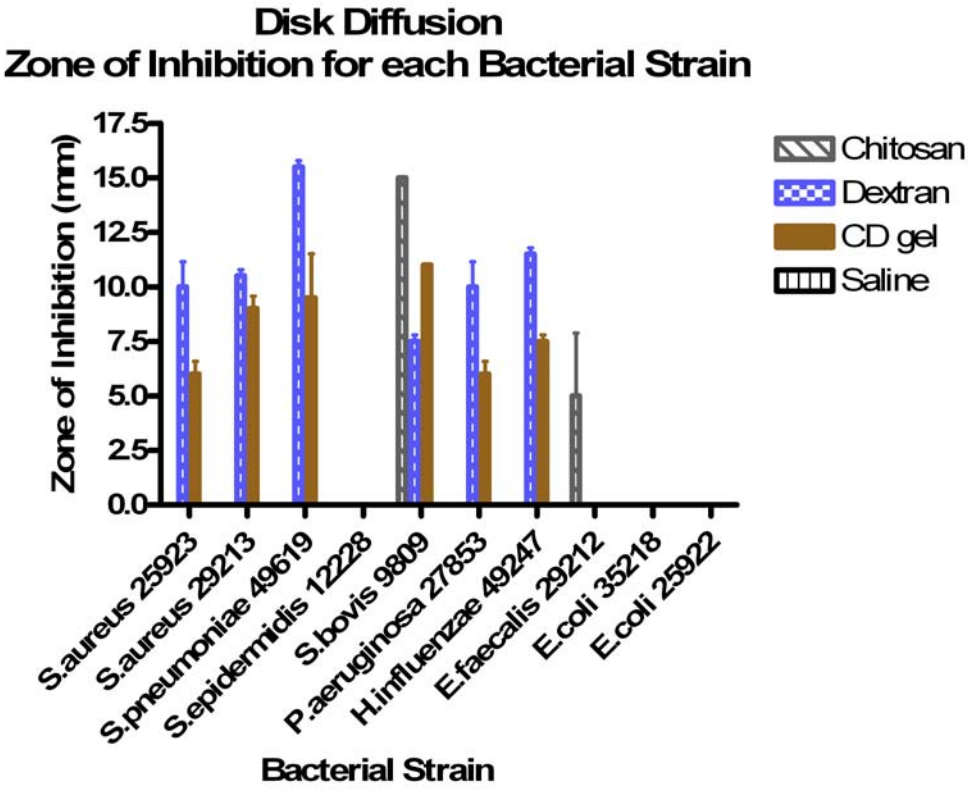
Graph 2.


Minimum Bactericidal Concentrations of agents for test bacteria
MBC (mg/ml)
50
40
30
20
10
0
Chitosan
Dextran
CD gel
S aureus 25923
S aureus 29213
S pneumoniae 49619
S epidermidis 12228
S bovis 9809
P aeruginosa 27853
H influnzae 49297
E faecalis 51299
E coli 35218
E coli 25922
Bacteria

DISK DIFFUSION METHOD

Growth inhibition as seen with disk diffusion method (**graph 3**) shows large zones of inhibition with dextran and CD gel for *S.aureus, S.pneumoniae, S.bovis*, *P.aeruginosa* and *H.influenzae*. In general, dextran had the greatest effect of growth inhibition with a slightly lesser inhibitory effect demonstrated by the CD gel. Chitosan only exhibited inhibition for *S.bovis* and *E.faecalis*.

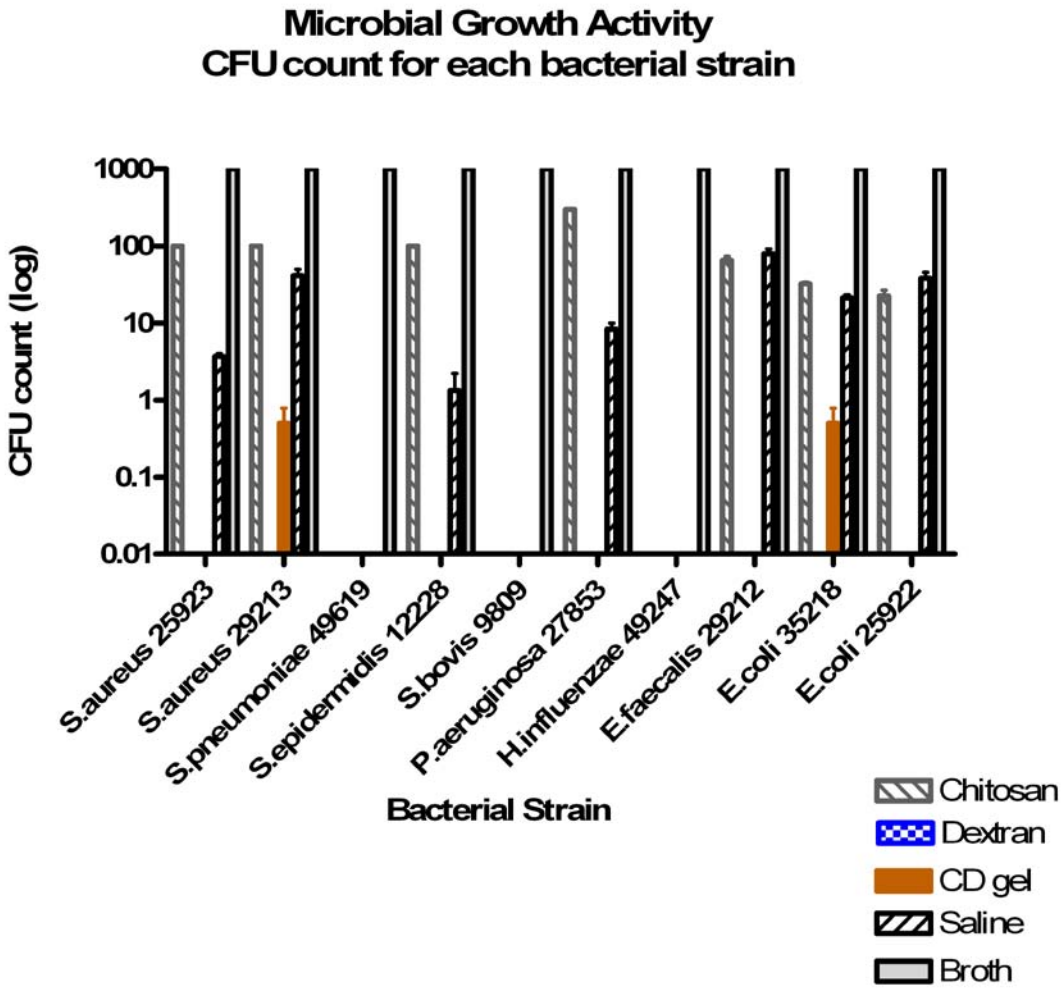
Graph 3.



MICROBIAL GROWTH ACTIVITY

Colony forming unit counts for each bacterial strain are shown in **graph 4.** In optimal growth conditions, there was no bacterial growth with all microorganisms inoculated into dextran. Similarly CD gel had only negligible growth with *E.coli* and *S.aureus* recording an average of less than 1 CFU in this group. Chitosan did not support growth of *S.pneumonia*, *S.bovis* or *H.influenzae* and had CFU's well below those of the positive growth control (1000 CFU). Interestingly, non-nutrient saline exhibited similar growth to chitosan.

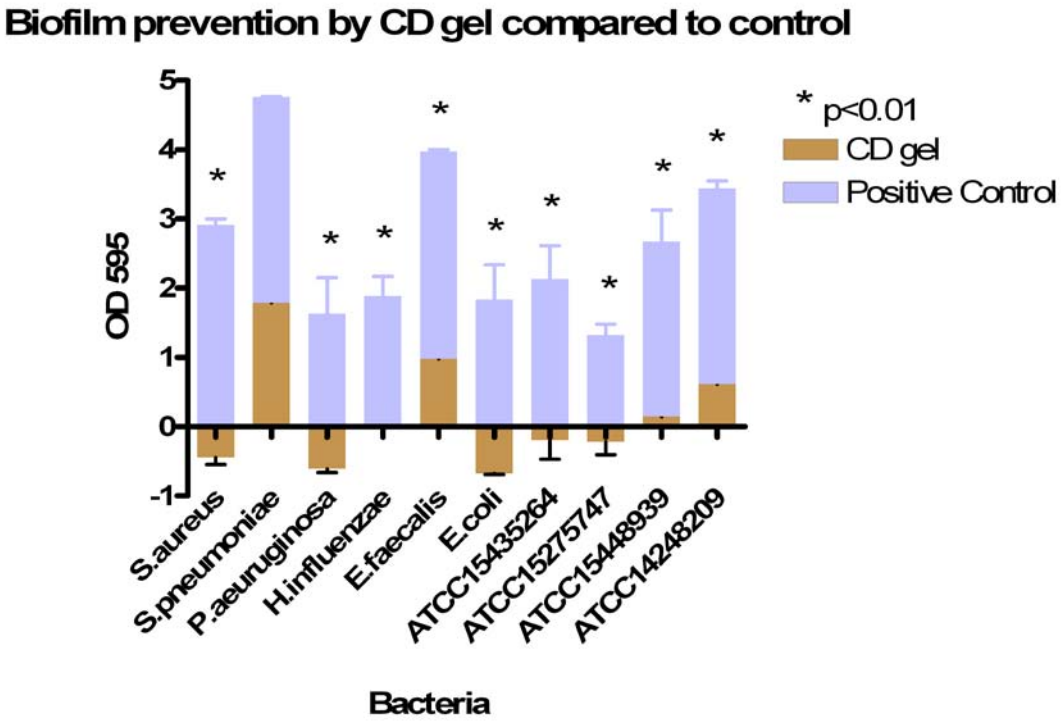
Graph 4.



IN-VITRO ANTI-BIOFILM PROPERTIES

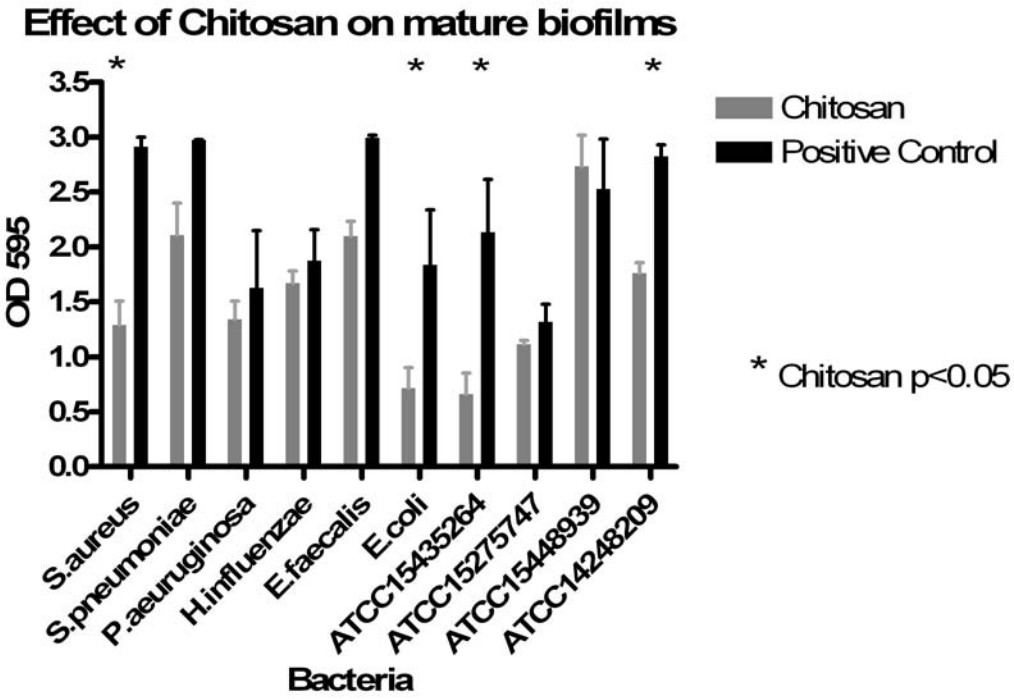
Effect of CD gel on biofilm formation is shown in **graph 5**. There was significant reduction in formation of biofilm for all bacteria tested ($p<0.01$), apart from *S.pneumoniae*, which had a non-significant reduction of only 40%.

Graph 5.

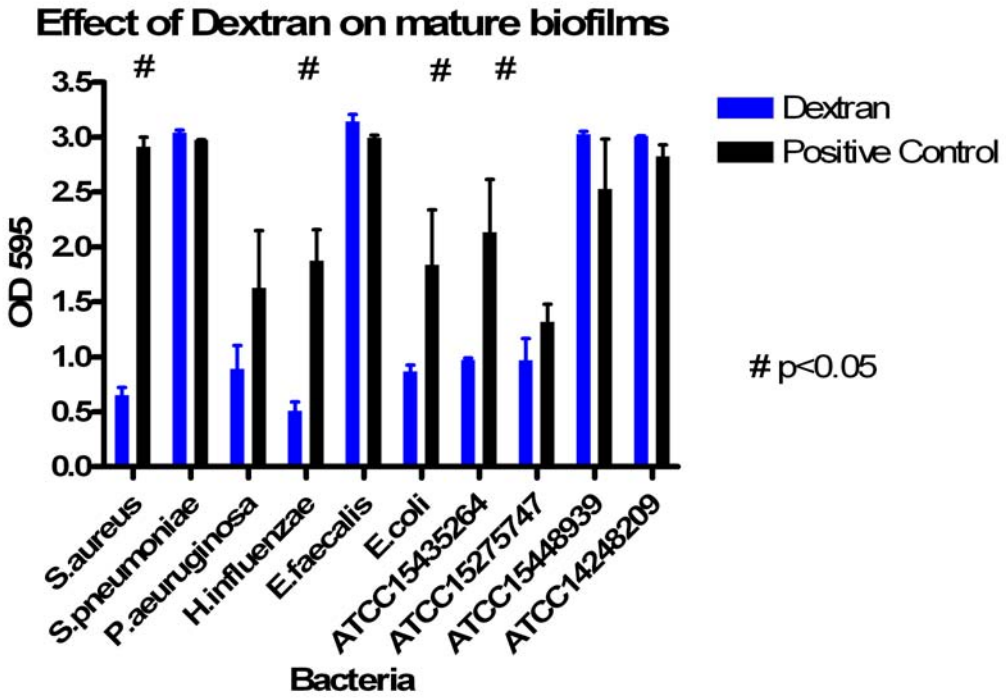


The effects of chitosan and dextran on formed mature biofilms can be seen in **graphs 6 and 7**. There was no significant increase in biofilm formation with either compound. Both chitosan and dextran significantly reduced biofilm presence for *S.aureus, E.coli*, ATCC 15435264 ($p<0.01$). In addition chitosan significantly reduced biofilm formation of ATCC 14248209 ($p<0.05$) and dextran significantly reduced biofilm formation of *H.influenzae* ($p<0.001$).

Graph 6. Effect of Chitosan on mature biofilms compared to positive control



Graph 7. Effect of Dextran on mature biofilms compared to positive control



DISCUSSION

Overall the findings of this in vitro study suggest that CD gel has marked antimicrobial activity both against planktonic and biofilm forming bacteria. It was inhibitory and bactericidal at subclinical concentrations (25mg/ml) for all bacteria tested apart from *E.coli* (50mg/ml) and when compared to a nutrient free environment (saline) as well as a positive growth control (broth) bacteria were essentially unable to grow in its presence. This supports randomised clinical trial data that found that sinonasal cavities sprayed with the mucosal adhesive CD gel had reduced appearance of infection compared to control[124].

Chitosan alone was the least potent of tested substances. It was however bactericidal to all bacteria tested at concentrations of 50mg/ml and compared to broth was significantly less supportive of bacterial growth. Chitosan did not increase biofilm formation and indeed reduced biofilm mass for a number of the bacteria tested.

Chitosan is thought to disrupt cell membranes as microbes settle on its surface[125]

This compares well to a similar study which used reduction assay and CFU determinates to investigate the damaging effect of chitosan on *Cryptococcus neoformans* biofilms[126].

Dextran was the most potent agent tested against planktonic bacteria with very low bactericidal concentrations required for most bacteria (apart from *P. aeruginosa* and *E.coli*). In addition it had wide zones of inhibition for most bacteria tested and did not support growth of any bacteria as seen in the CFU count (fig 4). Its effect on biofilms was variable with reduction in biofilm mass seen with similar bacteria to chitosan as well as with *H.influenzae.*

Dextran has been successfully used as a hydrogel coating for antifungal preparations and has been shown to penetrate the interior of biofilm cell clusters[127].

A limitation of this study occurred in the testing of the in-vitro anti-biofilm effects of CD gel, and its components, chitosan and dextran. Different study methodologies were applied to investigate the effect on biofilm formation with contact-exposure to the test agents. Although an overall trend towards reduction of biofilm growth could be observed with treatment by all test compounds, the methodology could not be replicated in dextran and chitosan, as it was for CD gel.

The components of CD gel, dextran and chitosan, prior to being combined, do not display the muco-adhesive properties afforded by CD gel, which allowed it to be smeared and rendered on the walls of the micro-wells of the 96-well plate. A reasonably consistent surface area for contact exposure to inoculated broth can be obtained in this way, and can also reduce the chance of removal of gel in the alternate daily media exchange procedure. Dextran and chitosan, are in aqueous states before they form a gel when combined, so it was not possible to achieve consistent contact surface area, because the addition of inoculated broth would then cause mixing.

Therefore investigation of the constituent parts, chitosan and dextran, continued independent of CD gel, using the same protocol described in a preceding *in vitro* anti-biofilm study [41].

The selection of bacterial strains in the in-vitro biofilm portion of the study was also considered a limitation. Only those bacterial strains that demonstrated biofilm-forming capacity on permanox chamber slide CLSM analysis, and a selection of clinically relevant strains isolated from CRS patients, were tested against the agents. A representative spectrum of clinically relevant bacteria, not identical to the group examined in the microbial susceptibility and MIC/MBC studies, was the result of this selection procedure.

CD gel has previously been shown to have unique abilities in being both a potent hemostatic agent as well as assisting in sinonasal wound healing [51, 52, 124, 128]. The presence of antimicrobial activity in addition to haemostatic and wound healing properties of this gel suggests a highly useful biomedical role.

CONCLUSION

Using standard measures of antimicrobial and antibiofilm activity we have investigated CD gel and its individual components - chitosan and dextran. Our results suggest CD gel has potent antibacterial and antibiofilm activity against a number of pathogenic organisms suspected of being involved in acute and chronic rhinosinusitis and therefore holds promise in its use as a nasal dressing.

Acknowledgments

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CHAPTER 4

SUMMARY

SUMMARY

The studies in this thesis were designed to assess the efficacy of potential anti-biofilm treatments. The trials provided opportunities to re-visit our evolving knowledge on the topic of bacterial biofilms and chronic rhinosinusitis, and to apply effective methods of biofilm detection and quantification. The well-established role of biofilms in the pathogenesis of CRS[9, 10, 12, 45] and their staggering resistance to conventional antimicrobials has compelled researchers to explore novel approaches to therapy[54, 59].

In the first study we used a standardized *in vivo* sheep frontal sinus biofilm model [17] to examine the impact of various proposed anti-biofilm treatments on *S. aureus* biofilms. In the examination of the mucosal specimens, CSLM confirmed the presence of biofilms stained with Baclight fluorescent nucleic acid probes (Invitrogen, Molecular Probes), while *S. aureus* PNA FISH probes (AdanDx, MA) were used to quantify the extent of biofilm involvement.

In the second study, *in vitro* biofilms of common CRS bacteria were used as a tool to evaluate the anti-biofilm properties of CD gel, a novel chitosan-based mucoadhesive dressing. The MIC's and MBC's of Chitosan, Dextran and the combined CD gel were also documented for various CRS pathogens, in their planktonic and biofilm modes of growth.

A sheep frontal sinus model of CRS was developed by our department in a previous study of biofilms[17] and was considered a reliable *in vivo* model for the investigation of anti-biofilm treatments. The ESS procedure in this sheep model used standard human endoscopic equipment to experimentally occlude the frontal sinus ostia, to allow for sinus inoculation with a standardized dilution of *S. aureus*. The previous study demonstrated consistent evidence of biofilm morphology in 100% of inoculated occluded sinuses on CSLM imaging[17]. In this study, an incubation period of 8 days was chosen as it represented the time of peak growth for *S. aureus* biofilms, according to *in vitro* data[55].

Although it is accepted that biofilms can be poly-microbial[12, 22, 45], *S. aureus* is one of the most commonly isolated micro-organisms from CRS patients and may play a role in the pathogenesis of the condition[105]. It is for this reason and its well-documented biofilm-forming capacity[80] that *S. aureus* (reference strain ATCC25923) was chosen as the target microbe for this intervention study.

A number of potential anti-biofilm therapies were evaluated by *in vivo* and *in vitro* methods. Mupirocin (ENT Technologies, VIC) flushes; chemical surfactant (Citric Acid Zwitterionic Surfactant, Medtronic, FL) with hydro-pressure (Hydro-debrider, HD, Medtronic, FL); gallium nitrate (Sigma Aldrich, NSW); saline flushes, and chitosan/dextran (CD) gel. These act via various pathways to disrupt the potential barriers of resistance in biofilms.

In the *in vivo* sheep study all sinuses receiving treatment showed a statistically significant reduction in biofilm surface area coverage compared to no treatment.

The most impressive results were obtained from mupirocin flushes. A significant reduction in biofilm coverage (7.51%) was observed 24 hours after a single dose of mupirocin, trending towards further reduction (5.87%) by the 8^{th} day of follow-up.

However, twice-daily mupirocin flushes over a period of 5 days reduced biofilms to negligible levels (0.84%) 8 days after the initial dose.

Sinuses that received an intra-operative stat dose of gallium nitrate also showed an initial reduction in biofilm surface area coverage to 16.2%, with sustained reduction effect by day 8 post-treatment (10.0% biofilm surface area coverage).

Although initially effective in reducing biofilms 24 hours after its administration, CAZS/HD failed to show persistent effects. Biofilms in this group re-generated to involve 21.95% of the mucosal surface area, approaching pre-treatment levels after 8 days. A plausible explanation for the re-accumulation of biofilms in this group may be that treatment by CAZS under hydrodynamic force is detrimental to the mucociliary function of the sinus mucosa. This was evident in the histological examination of the sinuses of New Zealand white rabbits exposed to CAZS solution, showing marked deciliation phenomenon, which required 6 days to normalize[129]. This, together with a residual biofilm load potentially too large for the innate immune system to clear, may result in regeneration of surviving biofilms. The response to *regular* treatment of the sheep sinuses with CAZS/HD was not assessed in our study. A possible correlation between biofilm reduction and duration of treatment was postulated with the repeated intensive use of mupirocin lavage. The same association could not be made for CAZS/HD treatment in this study. To achieve access, the hydrodebrider (HD) system used in the delivery of CAZS solution requires direct endoscopic visualization of the frontal sinus ostium. If regular

CAZS/HD regimes were to be tested, the repeated induction of anaesthesia and technical difficulty of repeated surgery would preclude it from being performed comfortably in the sheep.

There were obvious limitations to using the sheep model in the investigation of biofilms in CRS. Previously, sheep have been used extensively by our research department to study the histological effects of mucosal inflammation[50, 53]. Although the sheep have similar anatomical configuration of their sinuses as well as a similar ciliated mucosa, possible differences in the immunological response to bacteria has not been fully investigated. In this study, it was also assumed that the state of induced acute sinus infection and inflammation in sheep occurs as the pathological process of CRS in humans. The acute mucosal infection induced within the obstructed sheep frontal sinus, after an incubation period of 8 days, may not reflect the chronic inflammatory changes in muco-periosteum that exist in chronically infected human sinuses. Nonetheless, the sheep model allowed the study to be randomized and prospective in design, which in human subjects would be difficult to justify ethically.

The mucosal samples, collected for immuno-histological and FISH analysis, were randomly selected from the frontal sinuses because of size limitations and mechanical and logistical restrictions imposed by the hybridization process and sophisticated CSLM equipment. The 1cm x 1cm mucosal samples were small enough for stable fixation and hybridization assays on glass mounts (SnowCoat, Surgipath) and coverslips, as well as mounting onto the automated Leica SP5 Spectral CSLM stage. The chosen area of mucosal tissue was considered technically ideal for the subsequent recording and analysis of 100 random fields of view at 20x magnification. The mapping tool on the LAS-AF software

(Leica Microsystems) was able to perform this procedure and record the high-resolution images within a reasonable timeframe of 20 minutes per sample. On average, the total time involved to harvest and hybridize the fresh mucosal samples by FISH assay, ready for fluorescent microscopy, was 120 minutes. Because the specimens tested were randomly selected from the sinus, it was felt that they should give a fair representation of the overall infective process of the entire sinus. The sample sizes in each of the treatment groups were divided not only between treatments but also between time of harvesting the specimens. Of the possible 54 sinuses randomized to a treatment regime, 52 received the complete treatment course. With the available number of sheep granted for use in this project, the authors considered it important to evaluate the effects of treatment not only 24 hours after the first dose but also 8 days later, in order to assess biofilm re-growth. The duplication of each treatment group for follow-up evaluation halved the sample sizes, but provided valuable data that was highly relevant to the paradigm of biofilm-mediated infection. Furthermore, the smaller sample sizes allowed more treatment arms to be investigated in a standardized method. Despite this, all groups showed reductions in biofilm surface area coverage that were statistically significant compared to no treatment, with the most remarkable reduction in the group treated with twice-daily flushes of mupirocin for 5 days.

An assumption was made that the percentage of biofilm surface area coverage of mucosa equated to the degree of sinus disease. Surface area coverage included not only the intensely fluorescent staphylococci on CLSM, but also their self-produced surrounding exopolymeric matrix. Recording clinical data from the sheep and endoscopically assessing their sinuses would have added more information to our understanding of the syndrome of CRS, and would be considered in future extension of this work. To avoid the stresses of

anesthetic induction involved in this semi-invasive clinical examination, post-mortem digital photographic evaluations of sheep's harvested mucosa were carried out instead.

The recent development of a novel absorbable haemostatic chitosan gel (CD gel) prompted research into its antibacterial properties against known biofilm-forming bacterial strains. Its mucosal healing properties demonstrated in *in vivo* sheep studies and its ability to prevent post-operative infection in other animal studies [52, 121] makes CD gel stand out as a potentially effective post-ESS nasal dressing.

As part of the second study, well-established methods of *in vitro* biofilm growth[55] were revisited with the aim of demonstrating the susceptibilities of common CRS pathogens to this novel CD gel. Methods utilized in the first study, such as the detection of fluorescently labeled live biofilm bacteria and characteristic biofilm morphology, were used as evaluation tools in the second study.

Biofilm-forming CRS bacteria and their planktonic counterparts were exposed to clinical concentrations of chitosan, dextran and CD gel. Subsequently an initial anti-microbial profile for CD gel and its components was established.

According to the MIC and MBC data, CD gel was bacteriocidal and inhibitory at subclinical concentrations for all bacterial strains except for *E. coli*. This reflected similar findings in previous antibacterial research on low molecular weight chitosans [130]. However, dextran demonstrated remarkable growth inhibition across a large spectrum of bacterial test strains in the macro-dilution, disk-diffusion methods and in optimal growth conditions.

Chitosan demonstrated poor anti-bacterial action, with none of the bacterial strains being susceptible at subclinical concentrations. However when combined with dextran to form CD gel, the synergy of positively charged polysaccharides and high-molecular weight

carbohydrate produced significant bacteriocidal action, producing large zones of inhibition for *S. aureus, S. pneumoniae, S. bovis, P. aeruginosa and H. influenzae*. Importantly, chitosan alone did not support the growth of these potential CRS pathogens compared to positive growth controls.

To test the potency of CD gel and its constituents against biofilm-forming strains of bacteria, a model of *in vitro* biofilm growth was followed based on a previously described method[55]. The pathogens were first checked for biofilm-forming capacity by inoculating broth-containing Permanox (Nunc, Roskilde, Denmark) plastic chamber slides over an optimal growth period, then washed and stained with the Live/Dead cell viability (Invitrogen) kit using standard protocols. The chambers were assessed for biofilm presence under CLSM, and only those organisms that formed visible biofilms were included in analysis using the micro-titre plate crystal-violet assay described by O'Toole and Kolter [2].

From the reduced optical density of the stained biofilms, it was demonstrated that CD gel had significant anti-biofilm action against CRS bacteria. The susceptible groups of bacteria included four clinical isolates of *S. aureus*. Furthermore, CD gel did not support the growth of *in vitro* biofilms compared to no treatment.

Against mature biofilms, chitosan and dextran demonstrated variable anti-biofilm activity across the tested bacteria. The two components of CD gel significantly reduced biofilms formed by *S. aureus, H. influenzae* and one of the clinical isolates, and did not support biofilm growth in any of the remaining tested strains.

A reduction in bacterial growth in the presence of dextran at supra-MIC levels suggests that a high-carbohydrate environment is not conducive to the growth and development of

biofilms. The MIC and MBC of CD gel was well below safe and tolerable concentrations used in recent human clinical trials [131, 132]. More importantly, the data demonstrated that the use of CD gel and its components (dextran and chitosan) did not support planktonic or biofilm modes of bacterial growth. Coupled with its mucosal healing properties, CD gel's added anti-bacterial and anti-biofilm action against organisms commonly implicated in CRS posits it as an ideal post-ESS dressing.

One of the limitations of this study was in the application of chitosan and dextran to the 96-well microtitre plate, because these separate components do not possess optimal surface-adhesive properties. In the clinical setting, chitosan and dextran were not designed as separate therapeutic applications, being inherently non-mucoadhesive in these states. Therefore, the components could not be smeared onto the walls of the micro-titre wells, as CD gel was, to produce consistent coverage of the plastic walls upon which the biofilms were to attach. Hence, for chitosan and dextran, an alternative but previously established assay technique[55] was used to determine their anti-biofilm efficacy against matured biofilms. This involved using the same crystal violet staining and optical density analysis method. Thus, a comparison of CD gel to its constituents, chitosan and dextran, could not be made with regard to its *in vitro* anti-biofilm actions. However, it was shown that all treatments, irrespective of their adhesive properties, did not increase biofilm density compared to positive growth controls.

CHAPTER 5

CONCLUSION

CONCLUSION

Chronic rhinosinusitis is a highly prevalent and debilitating disease that places significant socio-economic burdens on the community. Recent evidence has demonstrated the presence of bacterial biofilms on the mucosa of affected patients[43, 45, 73], suggesting its role in the pathogenesis of the condition. Biofilms are also implicated in other chronic diseases such as otitis media, chronic tonsillitis and cholesteatoma. The persistence of *S. aureus* and *P. aeruginosa* biofilms has been associated with poorer prognosis following endoscopic sinus surgery. CLSM has been used as a tool to confirm biofilm presence in refractory CRS and in those patients who require repeated surgery[43].

Using nucleic acid probes (Invitrogen Molecular Probes) and species-specific PNA FISH techniques to detect biofilms on fresh mucosal samples, we have explored the anti-biofilm characteristics of various topical agents in a validated sheep model of CRS.

Topical anti-bacterial agents offer high mucosal killing concentrations and can be safely administered with lower serum concentration and hence lower systemic side effects.

In the sheep biofilm model of CRS, mupirocin was markedly potent as anti-biofilm agent, reducing mucosal biofilm coverage within 24 hours after a single dose, trending towards further reduction after 8 days of follow-up. The most impressive results were obtained with twice daily applications of mupirocin over a treatment period of 5 days. Biofilms in this treatment group were effectively eradicated to negligible levels.

The *in vitro* bacterial susceptibility data for Chitosan Dextran gel against a spectrum of biofilm-forming pathogens, including clinical isolates, were impressive for this novel chitin derivative. Together with its wound healing and haemostatic properties, CD gel

promises to be an ideal post-ESS packing material that would prevent post-operative infection.

A number of *in vitro* and *in vivo* experiments were conducted in this thesis to explore the efficacy of novel and exciting potential anti-biofilm therapies. Much anticipated future clinical trials of these agents, which have unique mechanisms of action, are currently underway in our research department.

REFERENCES

1. Jorgensen J.H., T.J.D., *Susceptibility Test Methods: Dilution and Disk Diffusion Methods*. 8th ed. Manual of Clinical Microbiology 8th Edition, ed. M.e. al. Vol. I. 2003, Washington: ASM Press.
2. O'Toole, G.A., Kolter, R., *Inititaion of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis.* Mol Microbiol, 1998. **28**(1): p. 449-461.
3. Benninger, M.S., et al., *Adult chronic rhinosinusitis: definitions, diagnosis, epidemiology, and pathophysiology.* Otolaryngol Head Neck Surg, 2003. **129**(3 Suppl): p. S1-32.
4. Brook, I., *Microbiology and antimicrobial management of sinusitis.* J Laryngol Otol, 2005. **119**(4): p. 251-8.
5. Bhattacharyya, N. and L.J. Kepnes, *The microbiology of recurrent rhinosinusitis after endoscopic sinus surgery.* Arch Otolaryngol Head Neck Surg, 1999. **125**(10): p. 1117-20.
6. Al-Shemari, H., et al., *Bacteriology of the sinus cavities of asymptomatic individuals after endoscopic sinus surgery.* J Otolaryngol, 2007. **36**(1): p. 43-8.
7. Bendouah, Z., et al., *Biofilm formation by Staphylococcus aureus and Pseudomonas aeruginosa is associated with an unfavorable evolution after surgery for chronic sinusitis and nasal polyposis.* Otolaryngol Head Neck Surg, 2006. **134**(6): p. 991-6.
8. Meltzer, E.O., et al., *Rhinosinusitis: Establishing definitions for clinical research and patient care.* Otolaryngol Head Neck Surg, 2004. **131**(6 Suppl): p. S1-62.
9. Ramadan, H.H., J.A. Sanclement, and J.G. Thomas, *Chronic rhinosinusitis and biofilms.* Otolaryngol Head Neck Surg, 2005. **132**(3): p. 414-7.
10. Ferguson, B.J. and D.B. Stolz, *Demonstration of biofilm in human bacterial chronic rhinosinusitis.* Am J Rhinol, 2005. **19**(5): p. 452-7.
11. Perloff, J.R. and J.N. Palmer, *Evidence of bacterial biofilms in a rabbit model of sinusitis.* Am J Rhinol, 2005. **19**(1): p. 1-6.
12. Sanclement, J.A., et al., *Bacterial biofilms in surgical specimens of patients with chronic rhinosinusitis.* Laryngoscope, 2005. **115**(4): p. 578-82.

13. Palmer, J., *Bacterial biofilms in chronic rhinosinusitis.* Ann Otol Rhinol Laryngol Suppl, 2006. **196**: p. 35-9.

14. Costerton, J.W., L. Montanaro, and C.R. Arciola, *Biofilm in implant infections: its production and regulation.* Int J Artif Organs, 2005. **28**(11): p. 1062-8.

15. Donlan, R.M. and J.W. Costerton, *Biofilms: survival mechanisms of clinically relevant microorganisms.* Clin Microbiol Rev, 2002. **15**(2): p. 167-93.

16. Costerton, J.W., G.G. Geesey, and K.J. Cheng, *How bacteria stick.* Sci Am, 1978. **238**(1): p. 86-95.

17. Ha, K.R., et al., *A sheep model for the study of biofilms in rhinosinusitis.* Am J Rhinol, 2007. **21**(3): p. 339-45.

18. Walters, M.C., 3rd, et al., *Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of Pseudomonas aeruginosa biofilms to ciprofloxacin and tobramycin.* Antimicrob Agents Chemother, 2003. **47**(1): p. 317-23.

19. Stoodley, P., et al., *Biofilms as complex differentiated communities.* Annu Rev Microbiol, 2002. **56**: p. 187-209.

20. Sutherland, I.W., *The biofilm matrix--an immobilized but dynamic microbial environment.* Trends Microbiol, 2001. **9**(5): p. 222-7.

21. Whitchurch, C.B., R.A. Alm, and J.S. Mattick, *The alginate regulator AlgR and an associated sensor FimS are required for twitching motility in Pseudomonas aeruginosa.* Proc Natl Acad Sci U S A, 1996. **93**(18): p. 9839-43.

22. Harvey, R.J. and V.J. Lund, *Biofilms and chronic rhinosinusitis: systematic review of evidence, current concepts and directions for research.* Rhinology, 2007. **45**(1): p. 3-13.

23. Davies, D.G., et al., *The involvement of cell-to-cell signals in the development of a bacterial biofilm.* Science, 1998. **280**(5361): p. 295-8.

24. O'Toole, G.A. and R. Kolter, *Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development.* Mol Microbiol, 1998. **30**(2): p. 295-304.

25. Davies, D.G. and G.G. Geesey, *Regulation of the alginate biosynthesis gene algC in Pseudomonas aeruginosa during biofilm development in continuous culture.* Appl Environ Microbiol, 1995. **61**(3): p. 860-7.

26. Gerke, C., et al., *Characterization of the N-acetylglucosaminyltransferase activity involved in the biosynthesis of the Staphylococcus epidermidis polysaccharide intercellular adhesin.* J Biol Chem, 1998. **273**(29): p. 18586-93.

27. Ma, L., et al., *Assembly and development of the Pseudomonas aeruginosa biofilm matrix.* PLoS Pathog, 2009. **5**(3): p. e1000354.

28. Shirtliff, M.E., J.T. Mader, and A.K. Camper, *Molecular interactions in biofilms.* Chem Biol, 2002. **9**(8): p. 859-71.

29. Baldassarri, L., et al., *Enterococcus spp. produces slime and survives in rat peritoneal macrophages.* Med Microbiol Immunol, 2001. **190**(3): p. 113-20.

30. Francois, P., et al., *Physical and biological effects of a surface coating procedure on polyurethane catheters.* Biomaterials, 1996. **17**(7): p. 667-78.

31. Yarwood, J.M., et al., *Quorum sensing in Staphylococcus aureus biofilms.* J Bacteriol, 2004. **186**(6): p. 1838-50.

32. Rice, K.C. and K.W. Bayles, *Death's toolbox: examining the molecular components of bacterial programmed cell death.* Mol Microbiol, 2003. **50**(3): p. 729-38.

33. Kreft, J.U., *Biofilms promote altruism.* Microbiology, 2004. **150**(Pt 8): p. 2751-60.

34. Bagge, N., et al., *Pseudomonas aeruginosa biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production.* Antimicrob Agents Chemother, 2004. **48**(4): p. 1175-87.

35. Palmer, J.N., *Bacterial biofilms: do they play a role in chronic sinusitis?* Otolaryngol Clin North Am, 2005. **38**(6): p. 1193-201, viii.

36. Seiberling, K.A., et al., *Superantigens and chronic rhinosinusitis: detection of staphylococcal exotoxins in nasal polyps.* Laryngoscope, 2005. **115**(9): p. 1580-5.

37. Hall-Stoodley, L., et al., *Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media.* JAMA, 2006. **296**(2): p. 202-11.

38. Costerton, J.W., *Cystic fibrosis pathogenesis and the role of biofilms in persistent infection.* Trends Microbiol, 2001. **9**(2): p. 50-2.

39. Chole, R.A. and B.T. Faddis, *Evidence for microbial biofilms in cholesteatomas.* Arch Otolaryngol Head Neck Surg, 2002. **128**(10): p. 1129-33.

40. Chole, R.A. and B.T. Faddis, *Anatomical evidence of microbial biofilms in tonsillar tissues: a possible mechanism to explain chronicity.* Arch Otolaryngol Head Neck Surg, 2003. **129**(6): p. 634-6.

41. Perkins, J., et al., *Bacterial biofilm presence in pediatric tracheotomy tubes.* Arch Otolaryngol Head Neck Surg, 2004. **130**(3): p. 339-43.

42. Lanza, D.C. and D.W. Kennedy, *Adult rhinosinusitis defined.* Otolaryngol Head Neck Surg, 1997. **117**(3 Pt 2): p. S1-7.

43. Psaltis, A.J., et al., *Confocal scanning laser microscopy evidence of biofilms in patients with chronic rhinosinusitis.* Laryngoscope, 2007. **117**(7): p. 1302-6.

44. Psaltis, A.J., et al., *The effect of bacterial biofilms on post-sinus surgical outcomes.* Am J Rhinol, 2008. **22**(1): p. 1-6.

45. Sanderson, A.R., J.G. Leid, and D. Hunsaker, *Bacterial biofilms on the sinus mucosa of human subjects with chronic rhinosinusitis.* Laryngoscope, 2006. **116**(7): p. 1121-6.

46. Cryer, J., et al., *Evidence of bacterial biofilms in human chronic sinusitis.* ORL J Otorhinolaryngol Relat Spec, 2004. **66**(3): p. 155-8.

47. Perloff, J.R. and J.N. Palmer, *Evidence of bacterial biofilms on frontal recess stents in patients with chronic rhinosinusitis.* Am J Rhinol, 2004. **18**(6): p. 377-80.

48. Oliveira, K., et al., *Rapid identification of Staphylococcus aureus directly from blood cultures by fluorescence in situ hybridization with peptide nucleic acid probes.* J Clin Microbiol, 2002. **40**(1): p. 247-51.

49. Chiu, A.G., et al., *An animal model for the study of topical medications in sinusitis.* Am J Rhinol, 2007. **21**(1): p. 5-9.

50. Rajapaksa, S.P., et al., *The effect of a hyaluronic acid-based nasal pack on mucosal healing in a sheep model of sinusitis.* Am J Rhinol, 2005. **19**(6): p. 572-6.

51. Valentine, R., et al., *The efficacy of a novel chitosan gel on hemostasis after endoscopic sinus surgery in a sheep model of chronic rhinosinusitis.* Am J Rhinol Allergy, 2009. **23**(1): p. 71-5.

52. Athanasiadis, T., et al., *Effects of a novel chitosan gel on mucosal wound healing following endoscopic sinus surgery in a sheep model of chronic rhinosinusitis.* Laryngoscope, 2008. **118**(6): p. 1088-94.

53. Rajapaksa, S., et al., *The effect of insulin-like growth factor 1 incorporated into a hyaluronic acid-based nasal pack on nasal mucosal healing in a healthy sheep model and a sheep model of chronic sinusitis.* Am J Rhinol, 2005. **19**(3): p. 251-6.

54. Desrosiers, M., Z. Bendouah, and J. Barbeau, *Effectiveness of topical antibiotics on Staphylococcus aureus biofilm in vitro.* Am J Rhinol, 2007. **21**(2): p. 149-53.

55. Ha, K.R., et al., *In Vitro Activity of Mupirocin on Clinical Isolates of Staphylococcus aureus and its Potential Implications in Chronic Rhinosinusitis.* Laryngoscope, 2008. **118**(3): p. 535-40.

56. Uren, B., A. Psaltis, and P.J. Wormald, *Nasal lavage with mupirocin for the treatment of surgically recalcitrant chronic rhinosinusitis.* Laryngoscope, 2008. **118**(9): p. 1677-80.

57. Cross, J.L., H.H. Ramadan, and J.G. Thomas, *The impact of a cation channel blocker (furosemide) on Pseudomonas aeruginosa PAO1 biofilm architecture.* Otolaryngol Head Neck Surg, 2007. **137**(1): p. 21-6.

58. Chiu, A.G., et al., *Baby shampoo nasal irrigations for the symptomatic post-functional endoscopic sinus surgery patient.* Am J Rhinol, 2008. **22**(1): p. 34-7.

59. Desrosiers, M., M. Myntti, and G. James, *Methods for removing bacterial biofilms: in vitro study using clinical chronic rhinosinusitis specimens.* Am J Rhinol, 2007. **21**(5): p. 527-32.

60. Bhandari, M., et al., *High and low pressure pulsatile lavage of contaminated tibial fractures: an in vitro study of bacterial adherence and bone damage.* J Orthop Trauma, 1999. **13**(8): p. 526-33.

61. Dirschl, D.R., et al., *High pressure pulsatile lavage irrigation of intraarticular fractures: effects on fracture healing.* J Orthop Trauma, 1998. **12**(7): p. 460-3.

62. Moussa, F.W., et al., *Disinfecting agents for removing adherent bacteria from orthopaedic hardware.* Clin Orthop Relat Res, 1996(329): p. 255-62.

63. Bullen, J.J., et al., *Iron and infection: the heart of the matter.* FEMS Immunol Med Microbiol, 2005. **43**(3): p. 325-30.

64. Banin, E., M.L. Vasil, and E.P. Greenberg, *Iron and Pseudomonas aeruginosa biofilm formation.* Proc Natl Acad Sci U S A, 2005. **102**(31): p. 11076-81.

65. Forsberg, C.M. and J.J. Bullen, *The effect of passage and iron on the virulence of Pseudomonas aeruginosa.* J Clin Pathol, 1972. **25**(1): p. 65-8.

66. Chitambar, C.R. and J. Narasimhan, *Targeting iron-dependent DNA synthesis with gallium and transferrin-gallium.* Pathobiology, 1991. **59**(1): p. 3-10.

67. Zhou, J., C. Elson, and T.D. Lee, *Reduction in postoperative adhesion formation and re-formation after an abdominal operation with the use of N, O - carboxymethyl chitosan.* Surgery, 2004. **135**(3): p. 307-12.

68. Yeo, Y. and D.S. Kohane, *Polymers in the prevention of peritoneal adhesions.* Eur J Pharm Biopharm, 2008. **68**(1): p. 57-66.

69. Carlson, R.P., et al., *Anti-biofilm properties of chitosan-coated surfaces.* J Biomater Sci Polym Ed, 2008. **19**(8): p. 1035-46.

70. Martinez, L.R., et al., *The use of chitosan to damage Cryptococcus neoformans biofilms.* Biomaterials, 2010. **31**(4): p. 669-79.

71. Bae, K., et al., *Effect of water-soluble reduced chitosan on Streptococcus mutans, plaque regrowth and biofilm vitality.* Clin Oral Investig, 2006. **10**(2): p. 102-7.

72. Gerasimenko, D.V., et al., *[Antibacterial effects of water-soluble low-molecular-weight chitosans on different microorganisms]*. Prikl Biokhim Mikrobiol, 2004. **40**(3): p. 301-6.

73. Psaltis, A.J., et al., *The effect of bacterial biofilms on postsinus surgical outcomes.* American Journal of Rhinology, 2008. **22**: p. 1-6.

74. Bjarnsholt, T. and M. Givskov, *Quorum-sensing blockade as a strategy for enhancing host defences against bacterial pathogens.* Philos Trans R Soc Lond B Biol Sci, 2007. **362**(1483): p. 1213-22.

75. Hentzer M, E.L., *Quorum sensing: a novel target for the treatment of biofilm infections.* Biodrugs, 2003. **17**: p. 241-250.

76. Pitt W, M.M., *Ultrasonic enhancement of antibiotic action on gram-negative bacteria.* Antimicrob Agents Chemotherapy, 1994. **38**: p. 2577-2582.

77. Rediske A, R.B., *Ultrasonic enhancement of antibiotic action on Escherichia coli biofilms: an in vivo model.* Antimicrob Agents Chemotherapy, 1999. **43**: p. 1211-1214.

78. Costerton J, E.B., *Mechanism of electrical enhancement of efficacy of antibiotics in killing biofilm bacteria.* Antimicrob Agents Chemotherapy, 1994. **38**: p. 2803-2809.

79. Wattanakaroon W, S.P., *Electrical enhancement of streptococcus gordonii biofilm killing by gentamicin.* Arch Oral Biology, 2000. **45**: p. 167-171.

80. Domenico, P., et al., *Activities of bismuth thiols against staphylococci and staphylococcal biofilms.* Antimicrob Agents Chemother, 2001. **45**(5): p. 1417-21.

81. Ha K, P.A., *A sheep model for the study of biofilms in rhinosinusitis.* American Journal of Rhinology, 2007. **21**: p. 339-345.

82. Ha, K.R., et al., *In Vitro Activity of Mupirocin on Clinical Isolates of Staphylococcus aureus and its Potential Implications in Chronic Rhinosinusitis.* Laryngoscope, 2007.

83. Sutherland R, R.B., KE Griffin, *Antibacterial activity of Mupirocin (Pseudomonic Acid), A new antibiotic for topical use* Antimicrob Agents Chemotherapy, 1985. **27**(4): p. 495-498.

84. J Capobianco, C.D., *Mechanism of mupirocin transport into sensitive and resistant bacteria.* Antimicrob Agents Chemotherapy, 1989. **33**(2): p. 156-163.

85. Hill, R., *The bioavailability of mupirocin in nasal secretions in vitro.* Journal of Clinical Pathology, 2002. **55**: p. 233-235.

86. van Rijen, M.M., et al., *Intranasal mupirocin for reduction of Staphylococcus aureus infections in surgical patients with nasal carriage: a systematic review.* J Antimicrob Chemother, 2008. **61**(2): p. 254-61.

87. Davis, S.C., et al., *Microscopic and physiologic evidence for biofilm-associated wound colonization in vivo.* Wound Repair Regen, 2008. **16**(1): p. 23-9.

88. Ishikawa, J. and T. Horii, *Effects of mupirocin at subinhibitory concentrations on biofilm formation in Pseudomonas aeruginosa.* Chemotherapy, 2005. **51**(6): p. 361-2.

89. Solares, C.A., et al., *Treatment of chronic rhinosinusitis exacerbations due to methicillin-resistant Staphylococcus aureus with mupirocin irrigations.* Am J Otolaryngol, 2006. **27**(3): p. 161-5.

90. R. Donlan, J.C., *Biofilms:Survival mechanisms of clinically relevant microorganisms.* Clinical Microbiology Reviews, 2002. **15**(2): p. 167-193.

91. Desrosiers M, M.M., *Methods for removing bacterial biofilms: In vitro study using clinical rhinosinusitis specimens.* American Journal of Rhinology, 2007. **21**: p. 527-532.

92. Bhandari, M.S., Emil, *High and Low Pressure Pulsatile Lavage of Contaminated Tibial Fractures: An In Vitro Study of Bacterial Adherence and Bone Damage.* Journal of Orthopedic Trauma, 1999. **13**(8): p. 526-533.

93. Dirschl DR, D.D., *High pressure pulsatile irrigation of fresh fractures: effects on fracture healing.* Journal of Orthopedic Trauma, 1998. **12**: p. 460-463.

94. Bullen JJ, R.H., *Iron and infection: the heart of the matter.* FEMMS Immunology Medical Microbiology, 2005. **43**: p. 325-330.

95. Kaneko Y, T.M., *The transition metal gallium disrupts Pseudomonas aeruginosa iron metabolism and has antimicrobial and antibiofilm activity.* The Journal of Clinical Investigation, 2007. **117**: p. 877-888.

96. Seiberling, K.A., L. Grammer, and R.C. Kern, *Chronic rhinosinusitis and superantigens.* Otolaryngol Clin North Am, 2005. **38**(6): p. 1215-36, ix.

97. Sasama, J., et al., *New paradigm for the roles of fungi and eosinophils in chronic rhinosinusitis.* Curr Opin Otolaryngol Head Neck Surg, 2005. **13**(1): p. 2-8.

98. Perez-Novo, C.A., et al., *Prostaglandin, leukotriene, and lipoxin balance in chronic rhinosinusitis with and without nasal polyposis.* J Allergy Clin Immunol, 2005. **115**(6): p. 1189-96.

99. Chiu, A.G., *Osteitis in chronic rhinosinusitis.* Otolaryngol Clin North Am, 2005. **38**(6): p. 1237-42.

100. Jorissen, M., et al., *Anatomical variations and sinusitis.* Acta Otorhinolaryngol Belg, 1997. **51**(4): p. 219-26.

101. Vining, E.M., *Evolution of medical management of chronic rhinosinusitis.* Ann Otol Rhinol Laryngol Suppl, 2006. **196**: p. 54-60.

102. Stammberger, H., *Endoscopic endonasal surgery--concepts in treatment of recurring rhinosinusitis. Part I. Anatomic and pathophysiologic considerations.* Otolaryngol Head Neck Surg, 1986. **94**(2): p. 143-7.

103. Brook, I., E.H. Frazier, and P.A. Foote, *Microbiology of the transition from acute to chronic maxillary sinusitis.* J Med Microbiol, 1996. **45**(5): p. 372-5.

104. Brook, I., *Bacterial infection and antibiotic treatment in chronic rhinosinusitis.* Clin Allergy Immunol, 2007. **20**: p. 147-62.

105. Lin, A. and N.Y. Busaba, *Staphylococcus aureus and endoscopic sinus surgery.* Curr Opin Otolaryngol Head Neck Surg, 2006. **14**(1): p. 19-22.

106. Brook, I., *The role of bacteria in chronic rhinosinusitis.* Otolaryngol Clin North Am, 2005. **38**(6): p. 1171-92.

107. Brook, I., *Bacteriology of acute and chronic sphenoid sinusitis.* Ann Otol Rhinol Laryngol, 2002. **111**(11): p. 1002-4.

108. Brook, I., *Bacteriology of acute and chronic frontal sinusitis.* Arch Otolaryngol Head Neck Surg, 2002. **128**(5): p. 583-5.

109. Brook, I., *Bacteriology of acute and chronic ethmoid sinusitis.* J Clin Microbiol, 2005. **43**(7): p. 3479-80.

110. Psaltis, A.J., Weitzel, E.K., Ha, K., Wormald, PJ, *The effect of bacterial biofilms on post-sinus surgical outcomes.*
Am J Rhinol, 2008. **22**(1): p. 1-6.

111. Chandra, R.K., et al., *Long-term effects of FloSeal packing after endoscopic sinus surgery.* Am J Rhinol, 2005. **19**(3): p. 240-3.

112. Wormald, P.J., et al., *A prospective single-blind randomized controlled study of use of hyaluronic acid nasal packs in patients after endoscopic sinus surgery.* Am J Rhinol, 2006. **20**(1): p. 7-10.

113. Orlandi, R.R. and D.C. Lanza, *Is nasal packing necessary following endoscopic sinus surgery?* Laryngoscope, 2004. **114**(9): p. 1541-4.

114. Athanasiadis, T., Beule, A., Robinson, B., Robinson, S., Shi, Z., Wormald, PJ., *Effects of a novel chitosan gel on mucosal wound healing following endoscopic sinus surgery in a sheep model of chronic rhinosinusitis.* Laryngoscope, 2008. **In press**.

115. Gerasimenko, D.V., et al., *Antibacterial effects of water-soluble low-molecular-weight chitosans on different microorganisms.* Applied Biochemistry and Microbiology, 2004. **40**(3): p. 301-6.

116. Kulikov, S.N., et al., *Effect of the molecular weight of chitosan on its antiviral activity in plants.* Applied Biochemistry and Microbiology, 2006. **42**(2): p. 224-8.

117. Roller, S. and N. Covill, *The antifungal properties of chitosan in laboratory media and apple juice.* Int J Food Microbiol, 1999. **47**(1-2): p. 67-77.

118. Sebastien, F., Stephane, G., Copinet, A., Coma, V., *Novel biodegradable films made from chitosan and poly(lactic acid) with antifungal properties against mycotoxinogen strains.* Carbohydrate Polymers, 2006. **65**: p. 185-193.

119. Decker, E.M., et al., *A synergistic chlorhexidine/chitosan combination for improved antiplaque strategies.* J Periodontal Res, 2005. **40**(5): p. 373-7.

120. Rabea, E.I., et al., *Chitosan as antimicrobial agent: applications and mode of action.* Biomacromolecules, 2003. **4**(6): p. 1457-65.

121. Burkatovskaya, M., et al., *Use of chitosan bandage to prevent fatal infections developing from highly contaminated wounds in mice.* Biomaterials, 2006. **27**(22): p. 4157-64.

122. O'Toole, G.A., Kolter, R., *Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, congergent signaling pathways: a genetic analysis.* Mol Microbiol, 1998. **28**: p. 449-461.

123. Benninger, M.S., Ferguson, B.J., Hadley, J.A., et al, *Adult chronic rhinosinusitis: definitions, diagnosis, epidemiology, and pathophysiology.* . Otolaryngol Head Neck Surg, 2003. **129**(S): p. 1-32.

124. Valentine, R., et al., *The efficacy of a novel chitosan gel on hemostasis and wound healing after endoscopic sinus surgery.* Am J Rhinol Allergy. **24**(1): p. 70-5.

125. Carlson, R.P., Taffs, R., Davison, W.M., Stewart, P.S., *Anti-biofilm properties of chitosan-coated surfaces.* J Biomater Sci Polym Ed, 2008. **19**(8): p. 1035-46.

126. Martinez, L.R., Mihu, M.R., Han, G., Frases, S., Cordero, R.J., Casadevall, A., Friedman, A.J., Friedman, J.M., Nosanchuk, J.D., *The use of chitosan to damage Cryptococcous neoformans biofilms.* Biomaterials, 2010. **31**(4): p. 669-79.

127. Takenaka, S., Pitts, B., Trivedi, H.M., Stewart, P.S., *Diffusion of macromolecules in model oral biofilms.* Appl Environ Microbiol, 2009. **75**(6): p. 1750-3.

128. Gustafson, S.B., et al., *Chitosan dressing provides hemostasis in swine femoral arterial injury model.* Prehosp Emerg Care, 2007. **11**(2): p. 172-8.

129. Chennupati, S.K., et al., *Effects of an LL-37-derived antimicrobial peptide in an animal model of biofilm Pseudomonas sinusitis.* Am J Rhinol Allergy, 2009. **23**(1): p. 46-51.

130. D.V. Gerasimenko, I.D.A., *Antibacterial effects of water-soluble low-molecular weight chitosans on different micro-organisms.* Applied Biochemistry and Microbiology, 2004. **40**(3): p. 253-57.

131. Valentine, R., P.J. Wormald, and R. Sindwani, *Advances in absorbable biomaterials and nasal packing.* Otolaryngol Clin North Am, 2009. **42**(5): p. 813-28, ix.

132. Valentine, R. and P.J. Wormald, *Nasal dressings after endoscopic sinus surgery: what and why?* Curr Opin Otolaryngol Head Neck Surg. **18**(1): p. 44-8.